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Enzymatic Oxygenation Of Non-newtonian Fermentations

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ENZYMATIC OXYGENATION OF NON-NEWTONIAN FERMENTATIONS

by

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ABSTRACT

The liquid-film resistance of the air bubbles controls the rate of oxygen transport to the medium in conventionally air-sparged fermentations. As a result of this constraint, the oxygen supply to non-Newtonian fermentations frequently fails to satisfy the peak demand of the culture. The in situ release of oxygen by enzyme action was studied as a novel way to increase the oxygen supply to non-Newtonian fermentations.

A practical technique, involving the infusion of hydrogen peroxide and catalase (HPC) solutions to the fermentor during the period of active growth of the culture, was developed. Aspergillus oryzae was used as the test organism in a defined medium of salts and simple carbon sources as the substrate. Extracellular protease, a non-growth associated enzyme, was used as an index of product formation.

Normal protease titres were obtained when HPC solutions were infused into the fermentor for periods up to 24 hours during the growth phase of the culture. Nitrate in the fermentation medium was shown to inhibit the activity of the catalase enzyme and was replaced by ammonium salts. During the enzymatic release of oxygen, air at a reduced rate (10% normal) was sparged into the fermentor contents to remove excess carbon dioxide.

Studies using a model system of glucose and glucose oxidase, in the presence of paper pulp (1%) to simulate mycelial solids, indicated oxygen transfer efficiencies of the HPC oxygenation technique close to 100% in some instances and better than 75% in all instances. Torque measurements of the rotating impeller shaft of the 14-litre bench fermentor, in conjunction with the oxygen transfer data, showed that oxygen transfer by the HPC technique was essentially independent of agitator-power input at the levels tested.

Data available from the studies were used to design two large scale fermentors of 20,000 gallons capacity - one utilizing a conventional aeration-agitation system, and the other utilizing a reduced aeration-agitation system supplemented by HPC oxygenation during the active growth phase of the culture. An estimated cost of HPC materials was not offset by the calculated reduction in the energy costs for agitation and aeration. The economic feasibility of the HPC oxygenation technique was examined with respect to the requirement for additional productivity.

ACKNOWLEDGMENTS

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CHAPTER 1: INTRODUCTION

The impetus for the research recorded in this thesis grew out of the author's previous experience at the Connaught Medical Research Laboratories, University of Toronto, during the years 1951-1955. While employed as a staff member at the penicillin plant, many of the problems associated with deep culture mycelial fermentations were experienced under practical operating conditions.

Of the many technical constraints imposed on large scale fermentations, perhaps none was more challenging or vexatious than the requirement for an adequate supply of oxygen to Penicillium chrysogenum during the peak demand period.

Recent developments in fermentation technology have opened the door to improved fermentor design, process control and a better insight into the basic respiratory response of microorganisms. In the instrumentation field, the most notable advance has been the availability of reliable electrodes to measure the dissolved oxygen level in the media. Many non-Newtonian problems still exist in fermentation systems, such as evidenced by Penicillium and other fungal species which produce a thick porridge-like mycelial mass which interferes with agitation and air-bubble dispersion to a significant degree. Such systems usually

have their greatest oxygen demand when oxygen transport is poorest and, indeed, the yield of some fermentations is measured by the extent that the peak oxygen demand has been satisfied.

No other facet of fermentation technology has received as much attention as aeration and oxygen transport. Of the several factors relating to the supply of oxygen to the liquid fermentation media, the liquid-film at the gas-liquid interface offers the controlling resistance to the transfer of oxygen. Any operation that decreases the liquid-film resistance and/or increases the total surface area of the air bubbles, will increase the supply of oxygen to the fermentation medium, assuming, of course, that other conditions remain substantially unchanged. Increased agitation rate, increased head pressure in the fermentor, increased aeration rate and increased oxygen concentration in the sparge gas are common techniques invoked to improve oxygen transport in fermentation systems.

In discussing the alternatives that are commonly used to meet oxygen demand in fermentations, Finn (1967) has raised the following question: "What are the opportunities to use radically different schemes for supplying oxygen demand such as oxygen enrichment of air, electrolysis of the media or the addition of hydrogen peroxide?". The latter suggestion was intriguing since oxygen liberated from hydrogen peroxide would appear initially as dissolved oxygen in the liquid phase - effectively by-passing the

liquid-film resistance of the air-bubble, which is the major barrier to oxygen transport. A hydrogen peroxide-catalase (HPC) system for oxygenation of a fermentation was envisioned as a potential source of instantly available oxygen in the liquid phase that could meet any demand in almost any kind of fermentation. Moreover, such a system ought to be amenable to precise control at any predetermined oxygen level.

It is perhaps unrealistic to assume that certain non-Newtonian fermentations, and, indeed, Newtonian fermentations, could use to advantage a HPC technique for oxygenation during the entire fermentation cycle. In practice, the enzymatic release of oxygen would probably be restricted to that period of the fermentation cycle when the oxygen demand is greatest and most difficult to meet by conventional techniques.

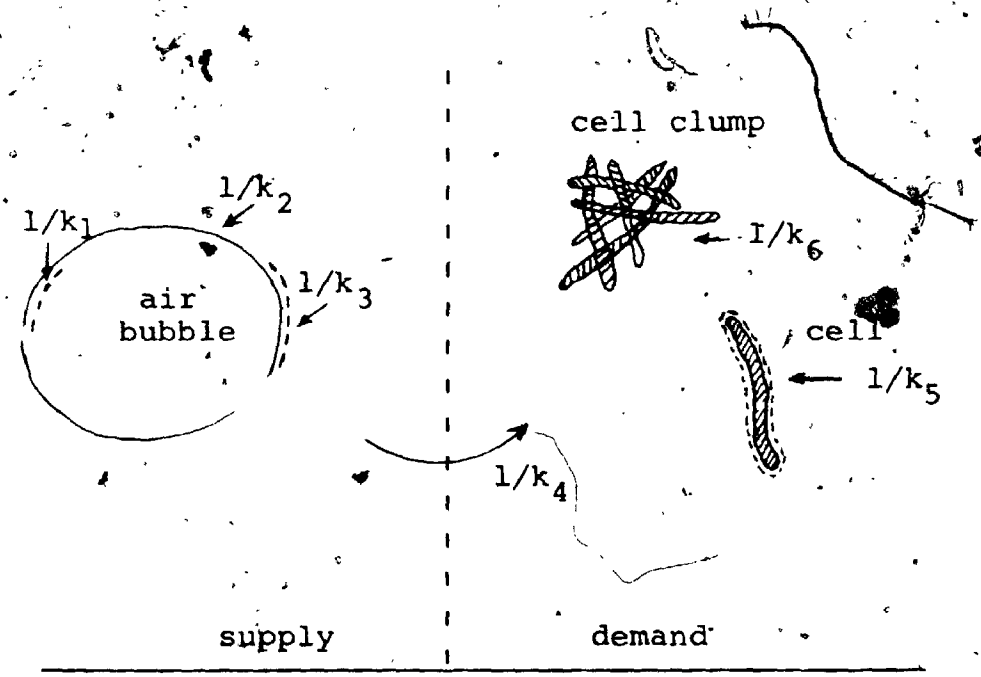
The researches detailed in this thesis examine the feasibility of meeting the oxygen demand of a non-Newtonian fermentation system by infusing hydrogen peroxide solutions to the fermentor during the period of active growth of the Aspergillus oryzae test organism in a defined medium of salts and simple carbon sources.

CHAPTER 2: LITERATURE REVIEW

Aeration, mixing, power input and scale-up in all their ramifications have preoccupied fermentation engineers for the past quarter century. The nature of these parameters as they relate to the fermentation industries are outlined in excellent review articles by Richards (1961), Finn (1967), Aiba (1965), Wang (1968), Oldshue (1966), Blakebrough (1964), and Calderbank (1967). It is abundantly clear that the application of sound engineering theory to fermentation practice has been the goal of the industry since classical anaerobic fermentations, namely alcohol production, have gradually yielded in importance to aerobic fermentations. At the present time, a great variety of economically viable fermentation products such as antibiotics, sterols, enzymes, organic acids, vitamins, alcohols, nucleotides, and proteins make a significant contribution to the gross national product of many countries of the world.

2.1 Concepts of Mass Transfer in the Aeration of Fermentation Media

The concern is primarily with oxygen transfer because most, though not all, fermentations are operated under conditions of oxygen limitation. Pirt (1966), has concluded from the comparison of diffusion constants of different nutrients that oxygen is most likely to be growth limiting in normal fermentation media. In other studies of mass transport, Bartholemew (1950a) listed the various resistances that are encountered by oxygen in the transfer from an air-bubble to the cell wall of a microorganism. Arnold and Steel (1958) and, somewhat later, Finn (1967) expanded this concept of oxygen transfer to include two groups of resistances: one group representing the supply side, the other the demand side. The individual resistances are indicated in Figure 1 taken from Finn.



$1/k_1$ - gas-film resistance

$1/k_2$ - gas-liquid interfacial resistance

$1/k_3$ - liquid-film resistance

$1/k_4$ - liquid-path resistance

$1/k_5$ - liquid-film resistance around cell

$1/k_6$ - intraclump resistance

Figure 1: Resistances to oxygen transfer

Of the series of resistances that are operative in the supply pathway for oxygen transfer to the fermentation media, the liquid-film resistance, '$1/k_3$' has been established by several independent researches as the rate-controlling resistance. Bartholemew (1950a) measured the temperature dependence of oxygen transfer from gas bubbles in cell-free systems. The results obtained indicated a temperature dependency of oxygen transport an order of magnitude greater than that which might be expected if the gas-film resistance were rate controlling. Calderbank (1958) using a light transmission technique to estimate the interfacial area of bubble dispersions concluded that the liquid-film diffusion coefficient was the major factor which influenced the rate of oxygen transfer. Yoshida et al (1960) were able to show from their studies of gas-liquid contractors that the volumetric mass transfer coefficient 'k_La' was the same for air and oxygen after allowances were made for partial pressure differences. This would clearly indicate that any resistance offered by the gas-film is negligible, since such resistance would be non-existant within a pure oxygen gas-film. Aiba (1961) came to a similar conclusion from his bubble absorption studies with a pure oxygen-water system.

On the demand side of the oxygen transport system, the intrinsic respiration processes of the organism, and not the liquid-film encompassing the cell, control the rate of oxygen uptake. Miura and Hirota (1966) came to this

conclusion from their aeration studies of yeast fermentations. They suggested that operating conditions throughout a fermentation cycle should be chosen to ensure a hyper-critical oxygen concentration in the bulk of the liquid medium. Borkowski and Johnson (1967) presented data to show that the thickness of the liquid-film around yeast cells was an inverse function of the degree of agitation up to a critical level which was reached when the power input was still relatively low. It was concluded that as long as the agitation level was sufficient to keep the cells suspended, the barrier to oxygen transport was of negligible consequence.

In viscous non-Newtonian fermentations, gas bubbles tend to be large and poorly dispersed; consequently direct transport of oxygen from the bubble to the cell would not appear to be a significant factor. Steel and Maxon (1966a) note that for non-Newtonian fermentation systems, respiration may be rate-limited by the resistance set up by clumps of cells.

Of the several theories offered in explanation of oxygen transport from the gas bubble to the fermentation medium, the two-film theory of Lewis and Whitman (1924) is the most widely accepted; though it is perhaps overly simplistic in its basic assumptions. Other theories, such as the "Penetration Theory" by Higbie (1935) and the "Surface Renewal Theory" by Danckwertz (1951) introduce oxygen diffusivity and a time dimension into the transport phenomenon. Regardless of the theory under consideration, all lead to the following general expression.

$$\frac{\text{Rate of transfer}}{\text{Unit area}} = \frac{N}{a} = (\text{Mass transfer coefficient})(\text{conc. gradient})$$

The two-film concept is illustrated in Figure 2 below.

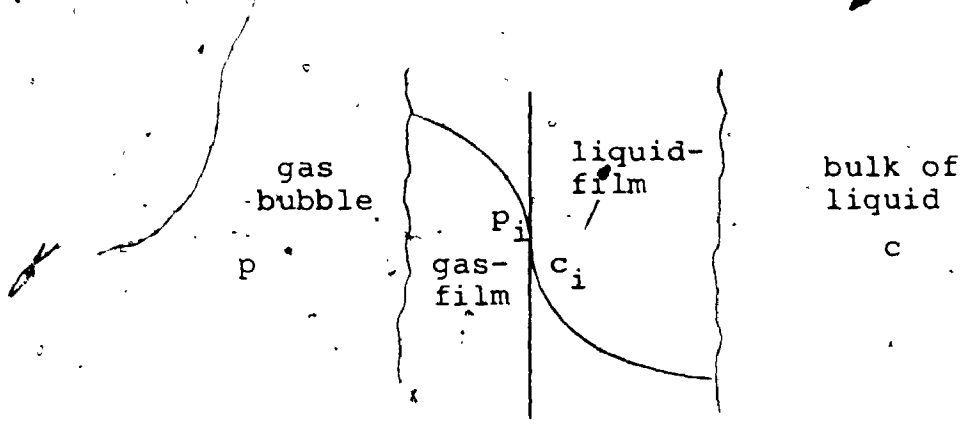


Figure 2: Two film concept of gas bubble

At equilibrium:

$$p_i = Hc_i \text{ at interface}$$

$$c^* = p/H$$

$$p^* = Hc$$

where

H = Henry's constant

p = oxygen partial pressure

c = oxygen concentration in the liquid

c^* & p^* = equilibrium concentration and pressure respectively.

At steady state:

$$\frac{\text{Rate of transfer}}{\text{Unit area}} = \text{transfer across gas film} = \text{transfer across liquid film} = \text{overall transfer}$$

or

$$\frac{N}{a} = k_g(p-p_i) = k_L(c_i-c) = K_g(p-p^*)$$

$$= K_L(c^*-c)$$

where K_g and K_L are overall mass transfer coefficients: units m/h

k_g and k_L are mass transfer coefficients: units m/h

"a" is bubble area/unit volume: units m^2/m^3,

It follows that

$$(c^*-c) = [(c^*-c_i) + (c_i-c)]$$

$$= \frac{K_L(c^*-c)}{k_g H} + \frac{K_L(c^*-c)}{k_L}$$

by dividing both sides of the equation by $K_L(c^*-c)$

$$1/K_L = 1/k_g H + 1/k_L = 1/K_g H$$

for a sparingly soluble gas such as oxygen

$$k_L \equiv K_L$$

$$\frac{N}{a} \equiv k_L(c^*-c)$$

or

$$N \equiv k_L a(c^*-c)$$

where $k_L a$ is volumetric transfer coefficient: units h^{-1}.

Under fermentation conditions, the equilibrium oxygen concentration c*, as determined by the gas phase, must of necessity be a mean value. The logarithmic mean value given below is the most useful.

$$(c^*-c)_{mean} = \frac{(c^*-c)_{inlet} - (c^*-c)_{outlet}}{2.3 \log \frac{(c^*-c)_{inlet}}{(c^*-c)_{outlet}}}$$

In a well-mixed fermentor $c^*_{mean} \equiv c^*_{outlet}$, thus a simple determination of the oxygen concentration in the exit gas and the liquid phase provides a reasonable estimate of $(c^*-c)_{mean}$.

Obviously, the rate that oxygen is transferred to the fermentation media is very much a function of the geometry of the fermentor and the operating conditions. Cooper et al. (1944) showed that k_La is a function of superficial air velocity 'V_s' and power per unit volume according to the following expression:

$$k_La = f[V_s (P/V)^{0.95}].$$

Calderbank (1958), on the other hand, was able to demonstrate a relationship between the bubble area per unit volume, superficial air velocity and power per unit volume as noted below:

$$a = f [V_s^{0.5} (P/V)^{0.4}].$$

Richards (1961) showed that the mass transfer coefficient k_L is a function of impeller speed N, and submitted the following expression for k_La variability:

$$k_La = f[V_s^{0.5}(P/V)^{0.4}N^{0.5}].$$

It is not surprising then that Steel and Maxon (1962) found that k_La correlated with impeller tip velocity for a series of impellers of different diameters which were used to agitate a non-Newtonian fermentation of novobiocin at constant power input. The smallest diameter impeller transferred oxygen at 8 times the rate of the largest diameter impeller at equal power input; however, oxygen transport in sulfite solution or for a yeast fermentation - both of which represent Newtonian systems - was shown to be independent of impeller diameter at constant power input. Blakebrough and Sambamurthy (1966) came to much the same conclusion upon examining mass transfer of oxygen in the presence of paper pulp suspensions in sulfite solution using impellers of different geometry. In the absence of pulp, the oxygen transfer was correlated to power input and not impeller speed for the different impellers examined, but in the presence of pulp (1.6%), impeller speed and impeller geometry, and not power input became important parameters of oxygen transport.

It appears that the shear rate of the impeller is an important variable that affects oxygen transport in non-Newtonian fermentations, but is relatively unimportant in

Newtonian fermentations of low viscosity; where inertial forces dominate the viscous forces in the turbulent regime.

2.2 Cell Respiration.

Internal respiration processes of the cell are enzyme mediated and appear to follow classical enzyme kinetic patterns. The Michaelis-Menten enzyme reaction hypothesis presupposes the formation of an intermediate enzyme-substrate complex which, in turn, breaks down to products and enzyme. In its simplest form the reaction can be written as follows:

enzyme + substrate $\rightleftharpoons$ enzyme-substrate complex $\longrightarrow$ enzyme + product

or

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}S \xrightarrow{k_3} E+P$$

The enzyme reaction velocity is given by the following expression:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where

$$K_m = \frac{k_2 + k_3}{k_1}$$

A typical plot of enzyme velocity against substrate concentration is indicated in Figure 3.

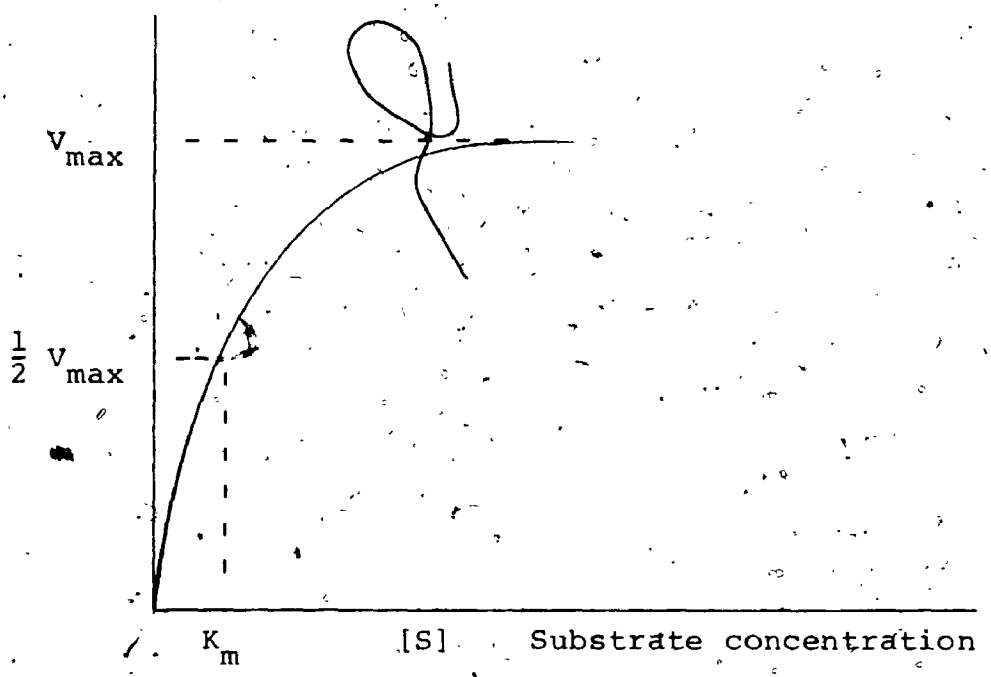


Figure 3: Typical plot of enzyme velocity versus substrate concentration

When the substrate concentration is above a critical level, the reaction rate is substantially independent of substrate concentration, thus characterizing a zero order reaction. When the substrate concentration is below the critical level, the reaction rate is dependent on substrate concentration, yielding to first order kinetics or those intermediate between zero and first order. Michaelis-Menten kinetics (Hixon and Gaden, 1950; Terui, 1968) have been applied to respiring systems, however, it should be noted that the Michaelis constant, so obtained, represents the entire system of respiring enzymes rather than a specific enzyme.

Fortunately for fermentation technologists, the critical oxygen concentration for most organisms is relatively low, thus one need not saturate the fermentation media with oxygen to establish maximum respiration rates. Finn (1954) has listed some critical oxygen concentrations (Table 1) for a sampling of organisms that have been reported by different investigators.

Table 1:

Critical oxygen concentrations for some organisms

Organism	Critical O_2, ppm
E. coli	0.26 @ 37.8°C
	0.10 @ 15.5°C
Yeast	0.12 @ 20°C
	0.15 @ 34.8°C
P. chrysogenum	0.70 @ 24°C
A. oryzae	0.64 @ 30°C

One of the most important considerations in aerobic fermentations is the maintenance of available oxygen in the bulk of the media at a concentration equal to or greater than the critical level in order that the respiration rate may proceed at its maximum velocity. Gaden (1956) and Finn (1954) have listed peak oxygen demands for a variety of organisms, some of which are reproduced in Table 2 below.

Table 2:

Peak oxygen demands of active cultures

Organism	Peak demand for oxygen
E. coli	0.013 mM O_2/g-min @ 30°C
Yeast	0.045 " @ 25°C
	0.16 " @ 30°C
S. griseus	0.017-0.050 " @ 27°C
Luminous bacteria	0.012 " @ 20°C
P. chrysogenum	0.5 mM O_2/litre-min
A. niger (citric)	0.47 mM O_2/litre-min
(amylase)	0.92 mM O_2/litre-min.
S. griseus	0.25 mM O_2/litre-min

It is not surprising that in many, if not most, aerobic fermentations, product yield correlates with the oxygen tension maintained in the fermentation media. Gaden (1959) characterized fermentations into three general types. In the first type, the product appears as a result of primary energy metabolism and is usually referred to as growth associated. This type of fermentation is exemplified in the production of yeast cell mass. In the second category, the fermentation product is indirectly keyed to cellular metabolism in which two rate maxima are in evidence: one that relates to cell

growth and the other to product formation. Citric acid production is an example of this type of fermentation. The third category gives rise to a main product which is independent of the main energy of metabolism and consequently is non-growth associated. Antibiotic synthesis, such as penicillin and streptomycin exemplify this type of fermentation.

The optimum oxygen tension for any particular fermentation is best established by trial and error. Some fermentations give the highest titres under conditions of continuously high aeration rates. Bartholemew et al (1950b) and Karow et al. (1953) illustrated this point with data from penicillin and streptomycin fermentations. Similar investigations (Roxburgh et al., 1954) concerned with the production of ustilagic acid by Ustilago zeae showed progressive yield increases up to an optimum aeration rate of 1.4 mM O_2/litre-min., as measured by sulfite oxidation tests. Zetelaki and Vas (1968) found that Aspergillus niger produced twice as much glucose oxidase activity when the fermentation was sparged with pure oxygen in lieu of air. In other published findings, Elsworth at al. (1957) discussed the effects of oxygen supply on the growth rate of Aerobacter cloacae. They reported exponential growth when oxygen was in excess of the critical value, but linear growth when oxygen was limiting. McDaniel (1965) established the dependency of E. coli biomass on oxygen supply in shake flask experiments.

There is evidence to suggest that the oxygen tension that is maintained in a fermentation materially alters the enzymatic make-up of the organism. Rolison (1952) came to this conclusion when comparing differences in oxygen demand of Penicillium chrysogenum cultured in shake flasks and in stirred fermentors. Blakebrough (1966) noted that the respiration rate of a culture of Neurospora crassa was permanently damaged when subjected to anaerobic conditions for three minutes. Carter and Bull (1971) examined the growth kinetics of Aspergillus nidulans and reported that mycelia grown at reduced oxygen tension had a lower critical oxygen concentration and a higher oxidative capacity than mycelia grown at high oxygen tension. These results suggest that other enzymes involved with oxidative metabolism are synthesized or activated in response to decreasing oxygen concentration in the fermentation media. An inductive effect of oxygen based on redox potential was suggested by Wimpenny (1969) to explain the phenomena. This conclusion was supported by Herbert (1965) who noted that cytochromes a_1 and a_2 were undetectable in Bacillus megaterium when grown under conditions of high oxygen tension. However, as oxygen tension was decreased, cytochromes a_1 and a_2 increased to a maximum, while cytochrome b remained relatively constant. Under anaerobic conditions or conditions of extremely low oxygen tension, the level of all three cytochromes decreased to low values.

It follows, therefore, that an optimum yield of a fermentation product does not necessarily require high oxygen tensions, but that intermediate levels are often preferable. Okada and Tsunoda (1965) have presented data to show how the ratios of α-ketoglutarate, glutamate and succinate changed with changing aeration rates of the fermentation media. Kempe and West (1959) noted that Lactobacillus delbrueckii, a microaerophilic organism, prefers a relatively low oxygen tension for the production of lactic acid. Highest penicillin titres were reported by Brown (1950) at intermediate aeration rates. It was reasoned that the higher aeration rates and the corresponding higher mycelial growth rates were responsible for excessive alkalinity, which, in turn, militated against penicillin production. Beker et al. (1973) described the physiological, morphological and biochemical response of Brevibacterium to different levels of dissolved oxygen tension in the fermentation menstruum. Virgilio (1964), in a simple but effective series of experiments, demonstrated the existence of a critical period with a high oxygen demand between the 50th and 80th hour of a 120 hour rifamycin fermentation. The high oxygen demand had to be satisfied during this critical 30 hour period to produce the required yield of antibiotic. If the oxygen demand was not satisfied during this critical period, regardless of the oxygen supply before or after, the yield of product was very poor. Sato (1961) referred to a similar critical period just before the onset of kanamycin production at about 50 hours into the fermentation cycle.

Both the rifamycin and kanamycin antibiotics are non-growth associated fermentation products and the demonstration of a favorable yield, when the oxygen demand was satisfied during a critical period, suggests the need to program the oxygen tension throughout the entire fermentation cycle. This is certainly well within the capability of current fermentation technology since recently available steam-sterilizable oxygen electrodes permit a continuous read-out and control function of dissolved oxygen concentration in most fermentations. As an extension to the need for optimum dissolved oxygen levels in fermentations there are clear indications that excessive aeration can lead to oxygen poisoning in specific instances. Sadoff et al. (1965) claimed that there was general inhibition of cultures of Pseudomonas fluorescens when oxygen concentrations above 6.5 ppm were maintained in the fermentation media; and Hromatka and Ebner (1949) reported that pure oxygen was harmful to Acetobacter in submerged vinegar fermentations.

Carbon dioxide poisoning is another fermentation phenomenon that has not been explored adequately by fermentation researchers. Nyiri and Lengyel (1965, 1968) drew attention to the inhibitory effects of carbon dioxide on the metabolism of Penicillium chrysogenum and the resultant excretion of penicillin. They examined the kinetics of carbon dioxide dissolution and dehydration and suggested that one of the necessary roles of aeration is to purge carbon dioxide from the fermentor. This operation was referred to as the

"ventilation of culture broths". Indeed, those who are familiar with mushroom production, are aware of the damaging effects of carbon dioxide (Rettew and Thompson, 1948) if allowed to accumulate in the growing rooms. Carbon dioxide levels over 1% favor production of mushrooms with long spindly stems and small heads and if the level exceeds 5%, mushrooms cease to form. Bennet and Kempe (1964) reported that the oxygen absorption of a sparged culture of Pseudomonas ovalis was approximately twice that of an unsparged system. This observation was explained in terms of the lower oxygen transfer resistance offered by cells on the bubble surfaces in sparged systems. It is interesting to speculate that this effect may be associated with excessive carbon dioxide accumulation which would materially reduce the respiration rate. In support of this possibility Bylinkina et al. (1973) have examined the effects of carbon dioxide tension on antibiotic production by different species of Actinomyces. It was reported that carbon dioxide concentrations greater than 20% saturation caused the respiration rate of the microorganism to decrease by about 40-50%. Specifically, the inhibition of the respiration rate of Actinomyces griseus, prior to the beginning of the biosynthetic process, markedly reduced the streptomycin titre.

To emphasize the complexity of carbon dioxide effects Dain et al. (1956) showed that a polysaccharide fermentation using different strains of Streptococcus bovis responded

favorably to a 5% carbon dioxide - 95% air mixture engulfing agar plates. Golding (1937, 1940) upon examining the colonial growth of Penicillium roqueforti under various ratios of air and carbon dioxide came to the conclusion that small concentrations of carbon dioxide in air increased the growth of the mold, whereas higher concentrations of carbon dioxide inhibited growth. The inhibition of growth was more apparent at low temperatures than at higher temperatures. Thus it seems clear that each fermentation has its own peculiar response to oxygen and carbon dioxide tension and that optimum operating conditions must be established in each case.

2.3 Bio-rheology

Fluids and suspensions in fluids are described as Newtonian or non-Newtonian according to their laminar flow behaviour when subjected to varying velocity gradients. Some typical shear diagrams for time independent flow models are shown in Figure 4 below.

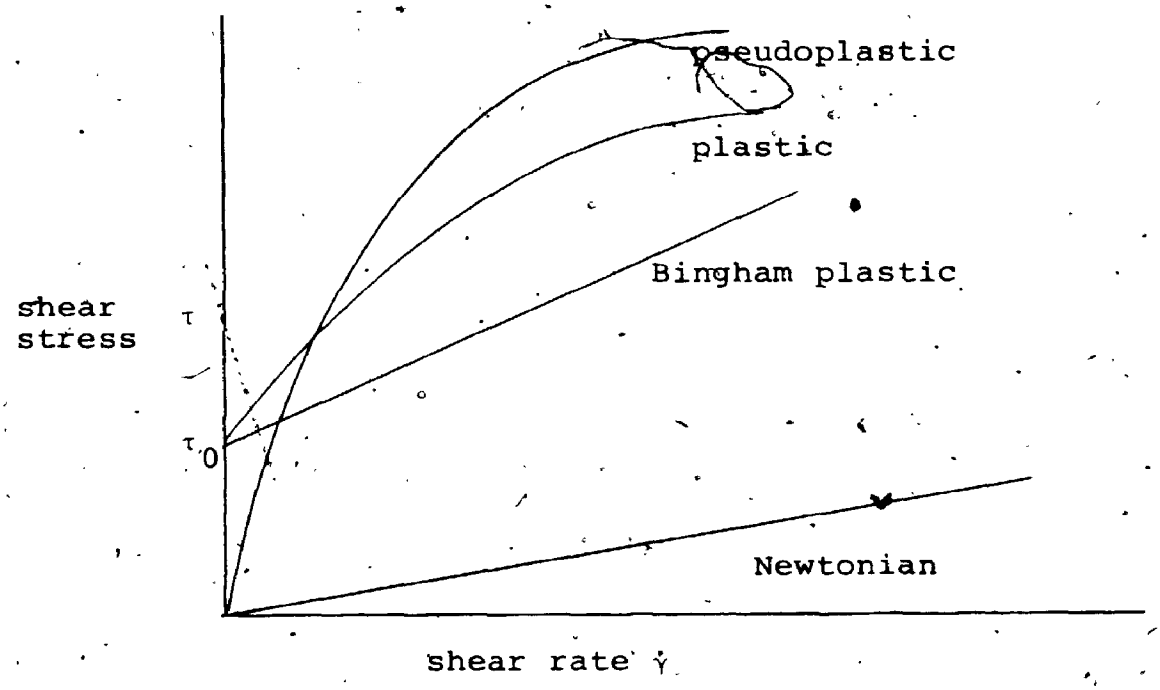


Figure 4: Shear diagrams for Newtonian and non-Newtonian fluids

The flow models are described by the following equations:

Newtonian $\tau = \mu\dot{\gamma}$

non-Newtonian: Bingham plastic $\tau = \tau_0 + \eta\dot{\gamma}$

pseudoplastic $\tau = K\dot{\gamma}^n$

plastic $\tau = \tau_0 + K\dot{\gamma}^n$

where

τ = shear stress, kg/m s^2, dynes/cm^2

μ = viscosity, kg/m s, g/cm s, poise

$\dot{\gamma}$ = shear rate, s^{-1}

η = coefficient of rigidity, kg/ms, g/cm s

K = consistency index, kg/m s^{2-n}, g/cm s^{2-n}

n = flow behaviour index.

Strictly speaking, all fermentations should demonstrate some degree of non-Newtonian flow behaviour since particulate matter suspended in the broth influences the hydrodynamics of the system; an influence that depends on the volume fraction and anisometric ratio of the particles and/or microorganisms. For all practical purposes, dilute suspensions of microorganisms of simple shape, non-filamentous and free from aggregation in chains can be safely characterized as Newtonian in flow behaviour. This classification would include most yeast and bacterial fermentations. On the other hand, fermentations with cultures of Penicillium, Aspergillus and Actinomyces exhibit marked non-Newtonian flow behaviour due to the physical nature of the mycelial solids.

The literature gives a somewhat confused picture of the specific type of non-Newtonian flow model that is represented by certain fermentations. Much of the published viscosity data are of questionable value because the authors failed to define their systems adequately in terms of appropriate parameters. For example, Deindoerfer and Gaden (1955) labelled their penicillin fermentation as a Bingham plastic and later Deindoerfer and West (1960) characterized the same fermentation as pseudoplastic. In a similar set of circumstances Solomons and Perkins (1958) described an Aspergillus niger fermentation as Bingham plastic, but later referred to it as a pseudoplastic fermentation (Solomons 1962). To add to the confusion, Steel and Maxon (1962) classified their novobiocin fermentation as a Bingham plastic.

It seems clear that most mycelial fermentations will fit the power law model for pseudoplastic flow behaviour. Bingham plastic or plastic flow behaviour may be incorrectly inferred when tests at low shear rates are not imposed on the system. Metzner and Otto (1957) using a pipe line flow analogy, suggested the following expression for apparent viscosity of non-Newtonian fluids that follow the power law model:

$$\mu_a = \frac{K}{(8N)^{1-n}} \left(\frac{3n+1}{4n}\right)^n$$

where μ_a = apparent viscosity

N = stirring speed

n = flow behaviour index

K = consistency index.

The above expression was modified by Calderbank and Moo-Young (1959) to include a proportionality constant of 0.8 to modify the consistency index. This led to better agreement of data on non-Newtonian fluids in stirred tanks which more properly related to classical fermentation situations. Thus for non-Newtonian fluids that follow the power law model it was possible to obtain an expression for a modified impeller Reynolds number by substituting the apparent viscosity 'μ_a' for the Newtonian viscosity 'μ' in the impeller Reynolds number formula.

$$N_{Re'} = \frac{D^2 N \rho}{\mu_a} = \frac{D^2 N^{2-n} \rho}{0.1\ K \left(\frac{6n+2}{n}\right)^n}$$

where

$N_{Re'}$ = modified impeller Reynolds number

D = impeller diameter

N = impeller velocity

ρ = fluid density

n = flow behaviour index

K = consistency index.

The flow behaviour index 'n' and the consistency index, 'K' are determined from the rheological behaviour of the non-Newtonian system. The power law model that describes pseudoplastic behaviour is as follows:

$\tau = K\dot{\gamma}^n$ where τ = shear stress, dynes/cm^2

$\dot{\gamma}$ = shear rate, s^{-1}

n = flow behaviour index

K = consistency index, g cm^{-1}s^{n-2}

The relationship between shear rate and spindle speed of the Brookfield viscometer was reported by Calderbank and Moo-Young (1959) to be:

$$\dot{\gamma} = \frac{4\pi N}{n} = b\ N$$

where

N = spindle speed, s^{-1}

$$b = \frac{4\pi}{n}$$

n = flow behaviour index

also

$\tau = \mu_a \dot{\gamma} = \mu_a bN$ where μ_a = apparent viscosity

$\therefore \quad \tau = \dot{\gamma}^n = K\, b^n N^n = \mu_a\, b\, N$

from which

$$K = \mu_a b^{1-n} N^{1-n}.$$

The flow behaviour index 'n' is obtained from the slope of the plot of log ($\mu_a N$) vs log N.

The constant 'b' is obtained by substituting values of n into the equation $b = \frac{4\pi}{n}$.

The consistency index 'K' is obtained by introducing a measured value for apparent viscosity at spindle speed N into equation

$$K = \mu_a b^{1-n} N^{1-n}$$

Data (Solomons, 1962) on the rheological behaviour of an Aspergillus niger fermentation demonstrated the effect of mycelial mass on the reduction of the degree of turbulence within a fermentor. For a consistency index, K = 172 cps, and a flow behaviour index, n = 0.35, modified Reynolds numbers that ranged from 21 to 413 were calculated for a variety of impeller diameters and speeds. These values compared dramatically with corresponding Reynolds numbers of 40,300

to 1,920,000 for similar systems using only water in the vessels. Thus an increase in the apparent viscosity, due to an increase in mycelial mass, leads to a considerable change in the flow regime of a non-Newtonian fermentation. In most instances, the relatively low concentration of mycelium during the lag and early growth phase does not present a serious mixing problem since the apparent viscosity is relatively close to the viscosity of the fermentation media prior to inoculation.

Steel and Maxon (1962) recorded apparent viscosities over the course of a Streptomyces niveus fermentation. It appeared that the apparent viscosity peaked at approximately 200 cps during the 4th day of the fermentation falling away sharply as the culture underwent some degree of autolysis towards the end of the cycle.

The non-Newtonian flow behaviour pattern of fermentations depends not only on the concentration but also on the shape of the culture organism. Einstein (1906, 1911), experimenting with dilute suspensions of rigid spheres, showed that the viscosity of mixtures could be predicted by the following equation:

$$\mu_m = \mu_0(1+2.5\phi)$$

where

μ_m = viscosity of mixture

μ_0 = viscosity of liquid without suspended solids

ϕ = volume fraction of suspended solids.

Vand (1948), some years later, modified Einstein's equation to encompass suspended solids of simple shapes. The spherical shape factor '2.5' was replaced by appropriate constants depending on the length to breadth ratio of the suspended solids. Vand's modification would seem to be more applicable to bacterial and yeast, rather than mycelial, fermentations. Randomly dispersed suspensions of mold in the filamentous form tend to immobilize a significant percentage of the suspending liquid, thus effectively increasing the volume fraction of the solid phase beyond the practical limitations of the modified Einstein expression.

In the above discussion on bio-rheology, the non-Newtonian flow behaviour of fermentations is induced solely by the suspended microbial solids and not by the fermentation medium. However, there is another important type of fermentation in which the non-Newtonian flow characteristics are attributable to the presence in the media of macro-molecules secreted by the culture organisms. The biosynthesis of polysaccharides and dextrans would be representative of this type of non-Newtonian fermentation. Benedict (1960) reported an apparent

viscosity of 800 cps. upon completion of a phosphomannan fermentation using Hansenula holstii as the culture organism.

2.4 Power Consumption in Fermentations

Rushton et al. (1950) established a correlation between the dimensionless power number and Reynolds number in stirred Newtonian fluids. Rushton's correlation which is given by

$$N_p = \text{constant } (N_{Re})^x$$

where

N_p = Power number

N_{Re} = Reynolds number

has been useful in scale-up operations for geometrically similar tanks. The exponent of the Reynolds number becomes -1 for $N_{Re} < 10$ (laminar flow) and becomes 0 for $N_{Re} > 10^4$ (turbulent flow). In the turbulent region, the Power number is a specific constant for each impeller and baffle design. For a fully baffled 6-bladed flat turbine impeller, the Power number is approximately 6.0.

Because the exponent 'x' in the Rushton correlation varies between -1 and 0, depending on the degree of agitation, the power requirements of the stirrer relate to density and viscosity in the following way:

$P = \text{constant } \mu N^2 D^3$ for $N_{Re} < 10$

$P = \text{constant } \rho N^3 D^5$ for $N_{Re} > 10^4$.

Power input to the impeller is, therefore, independent of density and dependent on viscosity for laminar mixing, and conversely, power is independent of viscosity and dependent on density for turbulent mixing.

Calderbank and Moo-Young (1959) extended Rushton's correlation to include non-Newtonian fluids that follow the power law model. The modified impeller Reynolds number, (Section 2.3) was introduced into the Rushton correlation and good agreement with experimental data was demonstrated.

Aerobic fermentations require large volumes of air causing agitation power to decrease substantially below that of the non-gassed contents. The power ratio of gassed to non-gassed media has been correlated by Ohyama and Endoh (1955) in the form of a dimensionless aeration number 'N_a' given by

$$N_a = \frac{Q/D^2}{N\ D}$$

where Q = volumetric flow rate, m^3/min

D = impeller diameter, m

N = impeller speed, s^{-1}

It follows from the above that the aeration number is proportional to the ratio of cross-sectional air velocity and impeller tip speed.

Michel and Miller (1962) introduced a correlation to show that the power for a gassed system was represented by the following relationship

$$P_g \propto \left(\frac{\rho^2 N D^3}{Q^{0.5}}\right)^{0.45}$$

and Nishikawa (1965) determined the proportionality constant for each of a series of non-Newtonian pseudoplastic systems representing different consistency and flow behaviour indices.

2.5 Oxygen Transport in non-Newtonian Fermentations

Most stirred fermentations are operated under conditions designed to give turbulent mixing of the tank contents. A somewhat anomalous situation arises since, as noted above, power input is independent of viscosity under turbulent flow conditions, yet the mycelial mass, which increases the apparent viscosity of the media, markedly attenuates the degree of turbulence within the fermentor. Tracer studies by Metzner (1960) of laminar and turbulent flow of Newtonian and non-Newtonian liquids in stirred tanks led him to the conclusion that most of the turbulent mixing takes place in the region of the impeller. Shear rates were found to decrease rapidly with increasing distance from the impeller. Transport studies on a series of gas-liquid systems by Wilhelm et al. (1966) indicated that under cell-free conditions, 40-50% of the oxygen absorption from air to water takes place in the impeller domain. Solomons (1961) came to a similar conclusion from his studies of oxygen absorption in the presence of mold mycelia; however, the implications are more serious. It was suggested that in viscous broths most of the

oxygen transport takes place within the impeller envelope with very little contribution from air bubbles outside the central core. Data on oxygen transport in Streptomycete fermentations, (Tuffile and Pinho, 1970), were consistent with the above conclusion. The poorest oxygen transport was obtained when the viscosity was highest.

Studies by Deindoerfer and Gaden (1955) demonstrated that killed mycelia of Penicillium, reconstituted in broth to 1.3% (dry basis), reduced the oxygen absorption coefficient by 85%. Brierley and Steel (1959) in a similar study reported a 90% and 85% reduction in the oxygen absorption coefficient for a 2% suspension (dry basis) of Aspergillus niger and a 2% cellulose pulp suspension, respectively. A 3% suspension of sago, however, did not lower the oxygen absorption coefficient significantly. Presumably, the predominately Newtonian nature of the sago suspension permitted uniform turbulent mixing with good air-bubble dispersion.

Paper-pulp suspensions undoubtedly provide a useful technique for the investigation of oxygen transport phenomena. Bugliarello and Daily (1961), in checking the rheological behaviour of fibre suspensions up to a concentration of 2%, concluded that they conformed to the pseudoplastic model rather than the Bingham plastic model. Studies by Bowers (1955) with paper pulp suspended in aqueous sulfite solution to simulate fermentation conditions, showed that oxygen

transport decreased by a factor of 20 in an unstirred fermentor and by a factor of 2 under high speed stirring. It was suggested that the gas bubbles were trapped by the paper pulp solids and coalesced into very large bubbles, effectively reducing the mass transfer area. Calderbank and Moo-Young (1961) concluded that small gas bubbles, < 2.5 mm in diameter, behave as rigid spheres and that large gas bubbles have somewhat higher transfer coefficients due to dynamic circulation within the walls of the bubbles. Surface contamination of the bubble interface, under most fermentation conditions, tends to reduce the higher transfer coefficients of the larger bubbles to that of rigid spheres. Deindoerfer and Humphrey (1961) presented data to show that the mass transfer rate for bubbles < 3 mm in diameter decreased with bubble size and that for bubbles > 3 mm in diameter a reasonably constant mass transfer rate existed. It was noted, however, that the longer a bubble existed in the liquid media, the lower its mass transfer coefficient became; this effect was attributed to surface contamination.

Reductions in oxygen transport in non-Newtonian fermentations that are related to the physical presence of the mycelia are aggravated by the introduction of antifoam agents. Phillips et al. (1960) and Solomons and Perkins (1958) published data to show that typical antifoam agents, silicones, lard oils, etc., can be expected to reduce oxygen transport by about 50%. Chain and Gualandi (1966) investigating the effect of antifoams on 3,000 litre penicillin fermentations

reported that these agents caused the oxygen concentration to drop from 50% saturation to zero, indicating that the available supply of oxygen could not meet the demand of the culture.

Insofar as mycelial fermentations are concerned, the weight of evidence suggests that when the oxygen demand is greatest, the physical conditions are least favourable for supplying oxygen to the organism. Above a certain basic minimum, increasing the aeration rate to the fermentor is of little value in increasing overall mass transfer of oxygen to the media in the presence of mycelia. Increasing the agitator speed is a more effective technique to improve oxygen transport, however, this is done at the risk of damage to the mycelia. Midler and Finn (1966) working with shear sensitive protozoa Tetrahymena pyriformis determined the fractional survival in a stirred fermentor under conditions of both laminar and turbulent shear fields. In these studies it was condluded that maximum shear stress close to the impeller, rather than the average shear stress in the body of the fermentor, was most disruptive to microorganisms; particularly mycelia. In non-Newtonian systems the cell damage was correlated with the impeller tip velocity, but not to Reynolds number or power per unit volume. There is, however, a physical constraint on the use of increased agitator speed to improve aeration efficiency since the power required to drive the agitator varies as the cube of the impeller speed.

Techniques such as increasing the fermentor head pressure, introducing pure oxygen or oxygen enriched air, are frequently used to improve the supply of oxygen to the culture without the risk of physical damage to the cells. A less practical approach was reported by Sadoff et al. (1956), who used electrolysis of the fermentation medium to provide oxygen for a culture of Pseudomonas fluorescens. A platinum anode discharged tiny bubbles of oxygen about 55μ in diameter. Cell yields equivalent to conventionally aerated cultures were obtained. It was of interest to note that, contrary to normal experience with air-sparged fermentations, the oxygen transfer was independent of power input in the turbulent mixing region, suggesting that the agitator was not required to produce small bubbles with correspondingly large total surface area for mass transfer. In spite of the obvious advantage of electrolysis for the production of small oxygen bubbles, the bulk of the oxygen must enter the media by diffusion through the liquid-film around each bubble.

2.6 Enzymatic Release of Oxygen

A somewhat novel technique for supplying oxygen to a fermentation culture is examined in this thesis. This technique involves the generation of oxygen in situ within the fermentation medium from a uniformly dispersed hydrogen peroxide - catalase (HPC) system. The salient feature of enzymatic oxygen release is that oxygen is generated in the dissolved state within the liquid medium, effectively eliminating the rate-controlling liquid-film resistance encountered in conventional aeration practices. It should be possible to maintain the oxygen tension at any predetermined level regardless of the demand of the culture provided that traces of hydrogen peroxide are not toxic to the organism. Foam problems would be minimal or non-existant and agitation power need only be sufficient to ensure adequate liquid-solid mixing. Because hydrogen peroxide and catalase are unstable when mixed, the technique would require a separate infusion channel for each reactant which would enter the fermentor at a rate predicated on the oxygen demand of the culture.

The literature has not drawn attention to the successful application of a HPC system as a general technique for supplying oxygen to fermentations. Some studies, however, have investigated the catalase activity of certain micro-organisms. Bruchmann (1966) reported that hydrogen peroxide increased the yield of citric acid by Aspergillus niger in submerged fermentation. The improved yield was explained,

not only in terms of increased oxygen supply through enzymatic decomposition of the peroxide, but also due to the specific action of undecomposed peroxide.

It is unlikely that catalase positive organisms could sustain the auto-enzymatic release of oxygen from hydrogen peroxide for protracted periods of time. Unpublished work of the author on the continuous fermentation of brewers' wort is a case in point. Wort containing 10 ppm hydrogen peroxide, to reduce the general level of infection in the substrate, was fed to the continuous fermentation system at a dilution rate of 0.04 h^{-1}. The fermentation proceeded with unabated vigor for one week after which the yeast rapidly succumbed to the debilitating effects of prolonged exposure to peroxide.

Catalase activities of yeasts and presumably all catalase positive organisms are regulated by inductive processes. Sulebele and Rege (1967) and Koyoma et al. (1968) examined the different levels of catalase activity in yeast relative to different conditions of growth and respiration. From their individual studies it would appear that catalase was induced by aeration and assumed a linear rate of production after an initial lag period. Catalase synthesis ceased when aeration was discontinued. It was also shown that hydrogen peroxide in dilute solution induced catalase activity in anaerobically grown cells; suggesting that peroxide, and not air, is the primary inducer. In any case,

it would be extremely risky, if not impossible, to rely on the fermentation culture to supply sufficient catalase activity for oxygen release from hydrogen peroxide, even for short intervals of time. A more positive approach would provide for a supplementary source of catalase to ensure the controlled release of oxygen from hydrogen peroxide without compromising any indigenous catalase activities of the organism.

2.7 Glucose - Glucose Oxidase System as a Measure of Oxygen Transport

The production of gluconic acid by a resting cell Pseudomonas ovalis fermentation of glucose was examined by Tsao and Kempe (1960) and Aiba et al. (1963) as a useful index of oxygen utilization. A simple acidometric titration was used to monitor the accumulation of gluconic acid, which, in turn, was related stoichiometrically to oxygen transport from the gas bubble to the liquid media.

Though the above biological technique for investigating oxygen transport phenomena is more representative of actual fermentation conditions than the classical sulfite oxidation method, the data could be misleading under certain test conditions. Humphrey and Reilly (1965) criticized the gluconic acid technique of Tsao and Kempe (1960) for measuring the rate of oxygen transport, insisting that d-gluconolactone accumulates as an intermediate

in the reaction and that gluconic acid production is, in fact, a measure of lactone hydrolysis. Tsao (1969) defended his previous position by pointing out that a waiting period of at least 30 minutes was programmed to ensure steady state production of gluconic acid prior to rate measurements. It was argued that steady state production of gluconic acid must reflect a corresponding steady state concentration of d-gluconolactone since the extracellular hydrolysis of this intermediate compound is able to proceed non-enzymically. Under these conditions the hydrolysis rate to gluconic acid would be a function of lactone concentration which, in turn, would be proportional to oxygen absorption into the test medium. Therefore, when lactone production reaches steady state, the production of gluconic acid is, indeed, proportional to oxygen transfer.

Hsieh et al. (1969) modified the gluconic acid technique for measuring oxygen transfer rates by replacing the resting cell suspension of Pseudomonas ovalis by a commercially available extract from Aspergillus niger. There was sufficient lactonase activity in their enzyme preparation to preclude significant accumulation of d-gluconolactone. Under these conditions, gluconic acid production was related quantitatively to oxygen transport.

There can be little doubt that the production of gluconic acid from glucose in the presence of a glucose oxidase extract is a useful technique for the investigation of oxygen transport phenomena and, indeed, was used herein to measure the efficiency of the HPC system as a source of biologically active oxygen for fermentations.

2.8 Addendum

A comprehensive review of recent studies on cell respiration was published by Harrison (1973). Data obtained from continuous culture techniques would suggest that cell respiration follows the general precepts of Michaelis-Menten kinetics, though the values of K_m and V_{max} are not strictly constant but may vary according to the fermentation conditions. For example, Carter and Bull (1971) reported that cultures of Aspergillus nidulans grown at high and low oxygen tension had different K_m values. Of special interest was their observation that the organisms demonstrated a sensitivity to dissolved oxygen tensions well above the K_m for the respiratory enzymes.

The effect of very high concentrations of carbon dioxide, (99.9%), on the growth rates of selected fungi, over extended periods of time, was examined by Calderon and Staffeldt (1973). During the first few weeks, carbon dioxide appeared to be fungistatic on cultures of Aspergillus niger and Aspergillus flavus but thereafter a restrained growth rate was observed. The altered cultural characteristics could be reversed by subculturing the fungi under aerobic conditions. Gandhi and Kjaergaard (1975) reported on the influence of carbon dioxide on the formation of α-amylase by cultures of Bacillus subtilis. Carbon dioxide concen-

trations, in the range of 3-8% in the inlet air, inhibited the growth rate but improved the cell and enzyme yield of the culture in batch fermentations. These results were explained in terms of a more efficient metabolism in the presence of low concentrations of carbon dioxide and suggest that the carbon dioxide tension in the culture media is an important fermentation parameter.

Oxygen transfer into mycelial pellets of Aspergillus niger was studied by Kobayashi et al (1973). The specific oxygen uptake rate was shown to be an inverse function of the pellet size, and, furthermore, the specific oxygen uptake rate was greater for mycelial matter removed from the outer region of the pellet as compared to the central region. The reduced respiration activity of the central region of the pellet could be restored to normal levels following a short incubation period (1-2 h) in a medium saturated with air.

In other studies, the effects of antifoam agents on oxygen transport from gas bubbles in sparged fermentation media were investigated by Benedek and Heideger (1971) and Yagi and Yoshida (1974). Both studies indicated that the marked reduction of oxygen transport in the presence of an antifoam agent is primarily due to the reduction in the specific surface area of the gas bubbles, rather than to changes in the mass transfer coefficient 'k_L' that might be attributable to differences in bubble diameter.

The measurement of the non-Newtonian viscosity of fungal fermentations by conventional instruments with rotating cylinders is not entirely satisfactory. Bongenaar et al (1973) suggested a more practical, though perhaps less fundamental, technique for characterizing the rheological properties of fermentation broths. Their technique involved a series of torque measurements at different turbine impeller speeds in a stirred sample of fermentor contents. These measurements permitted a direct calculation of the flow behaviour index 'n', however, the consistency index 'K' was obtained by comparison to a calibration fluid with a known consistency index value. Using the above technique, Roels et al (1974) examined the rheological behaviour of penicillin broths and concluded that the data does not fit the power law model satisfactorily at low shear rates. They suggested that the rheological behaviour of cultures of Penicillium could be explained in terms of a Bingham plastic model which incorporates a morphology or shape factor. This morphology factor is dependent on the ratio of the length to diameter of the mycelial hyphae. Leduy et al (1974) studied the rheological properties of a polysaccharide fermentation using a culture of Pullularia pullulans. In this fermentation the increase in apparent viscosity is largely attributable to the secretion of the soluble gums into the fermentation broth. The rheological behaviour changed from Newtonian to non-Newtonian early in the fermentation cycle and from non-Newtonian to Newtonian towards the end of the fermentation cycle.

Since the use of immobilized enzymes in flow reactors is an established unit process in biochemical engineering, several recent investigations have examined the stability of the immobilized enzymes in continuous contact with the substrate. Studies by O'Neill (1972), Altomare et al. (1974a), (1974b), indicated that the inactivation of immobilized catalase by hydrogen peroxide can be successfully predicted on the basis of first-order reaction kinetics. The inherent instability of catalase in the presence of hydrogen peroxide supports the requirement for the continuous metering of separate catalase and hydrogen peroxide solutions into the fermentation medium, when using the proposed hydrogen peroxide and catalase (HPC) oxygenation technique.

Information relating to the cost of agitation and aeration of mycelial fermentations is not readily available in the technical literature. Chain et al. (1966) assumed a power consumption of 1 watt/litre for aeration and this value was used by the author of this thesis to examine the economic feasibility of the HPC technique. Whitaker (1973) reported that the cost of agitation and aeration for a penicillin fermentation may vary from 19¢-29¢/h/1000 gal. for aeration rates of 0.06-1.0 V/V/min. These costs are in good agreement with the calculated costs for a conventional system of agitation-aeration that are detailed in Chapter 8.

CHAPTER 3: METHODS

3.1 Selection of the Fermentation System

The selection of the fermentation system to evaluate the efficiency of a hydrogen peroxide - catalase (HPC) technique for oxygenation was predicated on the following constraints:

1. The deep culture fermentation of the selected organism must be characterized as non-Newtonian with respect to its rheological properties.

2. The organism should be non-pathogenic, relatively stable and easy to manage by accepted microbiological techniques.

3. The fermentation cycle must be effected within a reasonable time frame using a defined medium that is inexpensive, uncomplicated, easy to prepare and readily duplicated.

4. The fermentation product should be a non-growth associated extracellular excretion product of the organism, that can be easily and accurately estimated by an established assay procedure.

5. The tolerance level of the culture and fermentation medium for hydrogen peroxide must be considerably greater than the maximum concentration likely to be encountered during the HPC infusion period.

For reasons of expediency it was decided to select an Aspergillus culture from several on hand in the culture collection of John Labatt Limited. These cultures originated from Japan and were obtained by the research department of the brewery as part of an investigation of the microbial production of malt replacement enzymes. The mycelial matrix of an Aspergillus culture in deep fermentation is representative of a non-Newtonian system with a peak oxygen demand that is difficult to satisfy.

Because cultures of Aspergillus are natural producers of amylolytic and proteolytic enzymes, the titre of extracellular protease in the fermentation broth was designated, albeit somewhat arbitrarily, as the index of product formation for the experimental fermentations reported in this thesis. Certainly, the production of an enzyme clearly identifies with a biological process and eliminates direct chemical involvement of the constituents of the fermentation medium. A simple and effective assay procedure was available to estimate protease formation during the course of the fermentation. The analytical method, first proposed by Kunitz (1947) is based on a measure of the amount of trichloracetic acid soluble products that are released from an enzyme digestion of casein.

It was not considered practicable to formulate a fermentation substrate that would maximize the yield of protease for the particular strain of Aspergillus involved

in the study. In all probability such a medium would be very complex and difficult to reproduce exactly on a continuing basis, not to mention the inordinate expenditure of time and effort that would have to be committed to approximate optimum fermentation conditions. A decision was made to select a defined fermentation medium from those previously published in the technical literature. Such a defined medium containing relatively simple carbon and nitrogen sources, could be easily reproduced and would permit a straightforward evaluation of fermentation parameters. Since extracellular protease was chosen, a priori, as the index of product formation, there remained the problem of selecting a specific Aspergillus culture and an acceptable fermentation medium to complete the system.

A series of shake flask experiments was carried out with five cultures of Aspergillus from the culture collection of the Labatt research department. The cultures were inoculated into each of several defined media culled from the literature. The object of this excercise was to "coarse-screen" the available cultures in different media and make a selection solely on the basis of growth response and protease titre.

Of the cultures examined, four were coded in the culture collection as Aspergillus oryzae 2630, 2633, 2608 and 2368 and the other culture was an uncoded strain of Aspergillus niger. The media utilized in screening were obtained by making minor variations of the standard Czapek-Dox medium (Dox, 1910)

listed below (Table 3). In addition, one formulation, reported by Tosoni and Glass (1963), containing lactic acid was tested.

Table 3.

Czapek-Dox fermentation medium

sucrose	30 g
$NaNO_3$	3 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4.7H_2O$	0.01 g
water	1000 ml

As reported by Raper and Fennell (1965) the Czapek-Dox medium with inorganic sources of nitrogen and chemically defined sources of carbon will permit moderately vigorous growth of most Aspergillus. The response was claimed to be relatively unaffected by minor variations in the medium, as for example, when glucose was substituted for sucrose and ammonium nitrate for sodium nitrate.

Shake flask tests were carried out in 300 ml Bellco flasks using 50 ml quantities of media adjusted to a pH in the 5.1-5.7 range. The reciprocating shake table was operated at 142 strokes per minute and the fermentation temperature was maintained at 28°C. The flasks were inoculated with 1 ml aliquots of culture washings from potatoe dextrose agar (Difco) slants. The flasks were observed for growth and assayed for protease at 45 and 86 hours.

Under the conditions of the test there was little doubt as to the choice of culture and medium. Only one of the five cultures in one specific medium gave a positive protease response after 86 hours on the shake table. Best results were obtained with Aspergillus oryzae 2608 in the fermentation medium described by Tosoni and Glass (1963). The ingredients of this medium are detailed in Table 4 below.

Table 4:

Basic fermentation medium

sucrose	7.2 g
glucose	3.6 g
KH_2PO_4	13.7 g
KNO_3	2.0 g
$MgSO_4$	1.23 g
lactic acid	3.54 g (neutralize c NH_4OH)
$Fe(NH_4)_2SO_4.6H_2O$	2 ppm
$ZnSO_4.7H_2O$	1.6 ppm
$MnSO_4.H_2O$	0.45 ppm
water	1000 ml
pH	5.2

It is entirely possible that given more favorable fermentation conditions, one of the other cultures or media might have shown more promise for the production of an extracellular protease, nevertheless, a practical fermentation system, that satisfied most of the criteria outlined in the beginning of this section, was available for further studies. It was decided to discontinue the search for other cultures and other fermentation media unless later investigations demonstrated an unsatisfactory tolerance level of the system to hydrogen peroxide.

A suitable sporulation agar was required to provide a reproducible source of spores for seeding the shake flask and deep culture fermentations. Three sporulation media, formulated specifically for cultures of Aspergillus, were evaluated for their ability to produce a good crop of spores with the selected culture. One sporulation agar, (Hasegawa, 1963), produced an excellent spore mat within a two-week period at ambient temperature. This formulation was adopted as the standard sporulation medium. The composition of this medium is shown in Table 5.

Table 5:

Sporulation agar

glucose	5 g
$NaNO_3$	10 g
$MgSO_4$	0.26 g
KCl	0.5 g
KH_2PO_4	90 g
$FeSO_4.7H_2O$	0.01 g
agar	30 g
water	1000 ml
pH	4.8

This sporulation medium is another example of a modified Czapek-Dox formulation. Since conidia do not develop until the carbohydrate source is consumed, the low sugar level in the medium favors the early onset of conidia formation. The large quantity of potassium phosphate ensures that the medium is well buffered on the acid side and the chloride ions promote the development of green conidia.

3.2 Specifications of Fermentation and Ancillary Equipment

3.2.1 Bench-top fermentor

manufactured by Fermentation Design Inc., Allentown, Pa.

specifications:

(i) vessel

material	pyrex glass jar
inside height	45 cm
inside diameter	21 cm
max. working volume	14 litres

(ii) stirrers

material	stainless steel
type	six-bladed flat turbine impellers
number of impellers	2
impeller dimensions	8.9 cm diameter, 1.7 cm wide
impeller location on stirring shaft	8.9 and 24 cm from the bottom of the fermentor jar

(iii) baffles

material	stainless steel
type	strips, 44×1.9 cm
number of baffles	4
location	90° apart, clearing sides and bottom of jar by 0.6 cm

(iv) aeration sparger

material	stainless steel
type	nozzle
location	centrally, below bottom impeller

3.2.2 Incubator shake table

manufactured by New Brunswick Scientific Co.
New Brunswick, N.J.

specifications:

stroke	gyrotory motion, 2.5 cm circular orbit
speed range	40-400 cycles per min
platform size	45×76 cm
number of stations	40
flask type	300 ml Bellco, conical
temperature control	ambient to 60°C

3.2.3 Dissolved oxygen measurement system

Beckman polarographic, model 778 oxygen analyser

Kent strip recorder - Multelec MK III 0-50 mV.

3.2.4 Infusion pump

manufactured by Harvard Apparatus Co.
Millis, Mass.

2 channel peristaltic, model 1201

3.2.5 Torque meter.

Manufactured by Baldwin Lima-Hamilton
Waltham, Mass.

type A-20 torque pick-up, 0-20 inch-pounds

3.2.6 Viscosity meter

manufactured by Brookfield Engineering Laboratories
Stoughton, Mass.

Syncro-lectric viscometer, model LVF

3.3 Analytical Procedures

3.3.1 Protease assay

Principle: Protease and casein substrate are incubated at 37°C. The liberated tyrosine is reacted with Folin reagent producing a color complex, the intensity of which is proportional to enzyme activity.

Reagents: (i) Phosphate buffer, pH 7.0

4.17 g $NaH_2PO_4.H_2O$ and 7.73 g Na_2HPO_4 were made up to 1 litre with distilled water.

(ii) Casein substrate

2 g of casein (Fisher Scientific cat. No. C-202) were added to 75 ml of phosphate buffer. The suspension was heated in a water bath to solubilize the casein. The solution was cooled to room temperature and diluted to 100 ml with distilled water.

(iii) Trichloracetic acid solution

A 0.4 molar solution of trichloracetic acid (TCA) was prepared in distilled water.

(iv) Folin reagent (Folin & Ciocalteau)

10 ml of stock Folin reagent (Fisher Scientific cat. No. So-p-24) were diluted to 50 ml with distilled water and stored in a dark place.

(v) Sodium carbonate solution

A 0.4 molar solution of Na_2CO_3 was prepared in distilled water.

Test procedure: The details of this enzyme assay were developed by Western Biochemical Corp., San Francisco, for their Pacific Protease. The sample for protease assay was filtered through Whatman #4 filter paper and the resultant filtrate was appropriately diluted in phosphate buffer to give an enzyme activity that would liberate 1-20μg/ml of tyrosine under the test conditions. 2 ml of the diluted enzyme solution were pipetted into a test tube and placed in a 37 ± 0.1°C water bath. Then 2 ml of casein substrate, previously heated to 37°C were added to the enzyme solution and the contents were thoroughly mixed. The mixture was left to incubate at 37°C for exactly ten minutes, at which time 4 ml of TCA solution were added to destroy all enzyme activity. The contents of the test tube were incubated for an additional twenty minutes, then filtered through Whatman #4 paper. 1 ml of the filtrate from the enzyme digestion was added to 5 ml of Na_2CO_3 solution in another test tube. 1 ml of the diluted Folin reagent was pipetted into the test tube and the contents were thoroughly mixed. Following a twenty minute incubation period at 37°C for full color development, the color intensity was measured by a Klett colorimeter with a #62 red filter. A blank determination was run on each assay sample. The procedure was identical to that of the active protease sample except that the 4 ml of TCA solution were added to the enzyme solution prior to the addition of the casein substrate. The Klett colorimeter was zeroed on the blank.

Preparation of the standard curve: A stock solution of ℓ-tyrosine (Nutritional Biochemicals Corp., Cleveland, Ohio) containing 20µg/ml was prepared in 0.2 M TCA solution. Appropriate dilutions of this stock solution were made in 0.2 M TCA solution to give a range of tyrosine concentrations from 0-20µg/ml. 1.0 ml of each standard dilution was pipetted into 5 ml of Na_2C0_3 solution and thoroughly mixed. Folin reagent was added and the color intensity was developed and measured on the Klett colorimeter as indicated above. The Klett readings for the range of tyrosine concentrations are listed in Table 6 and are plotted in Figure 5.

Table 6:

Standard tyrosine versus Klett readings

Tyrosine µg/ml	Klett reading
0	0
2	11
4	21
6	31
8	42
10	53
12	64
14	75
16	84
18	96
20	107

Definition of a protease unit: One unit 'U' of protease activity is that quantity of enzyme releasing one micro equivalent of TCA soluble tyrosine per minute under the test conditions.

Calculation of protease activity:

$$\text{protease units/ml} = \frac{\mu\text{g tyrosine/ml} \times \text{sample dilution} \times \text{assay dil.}}{10 \text{ min} \times 181 \text{ M.W. tyrosine}}$$

or, since standard curve is linear,

$$\text{protease units/ml} = \frac{\text{Klett rdg} \times \text{sample dilution} \times \text{slope}^{-1} \text{ of standard curve}}{10 \times 181}$$

$$\text{protease units/ml} = \text{Klett rdg} \times \text{sample dilution} \times 0.00041$$

For convenience all assay results are reported in milli-units 'mU' of protease activity. A replication (ten samples) of this protease assay indicated a standard deviation equal to 2.2% of the mean value.

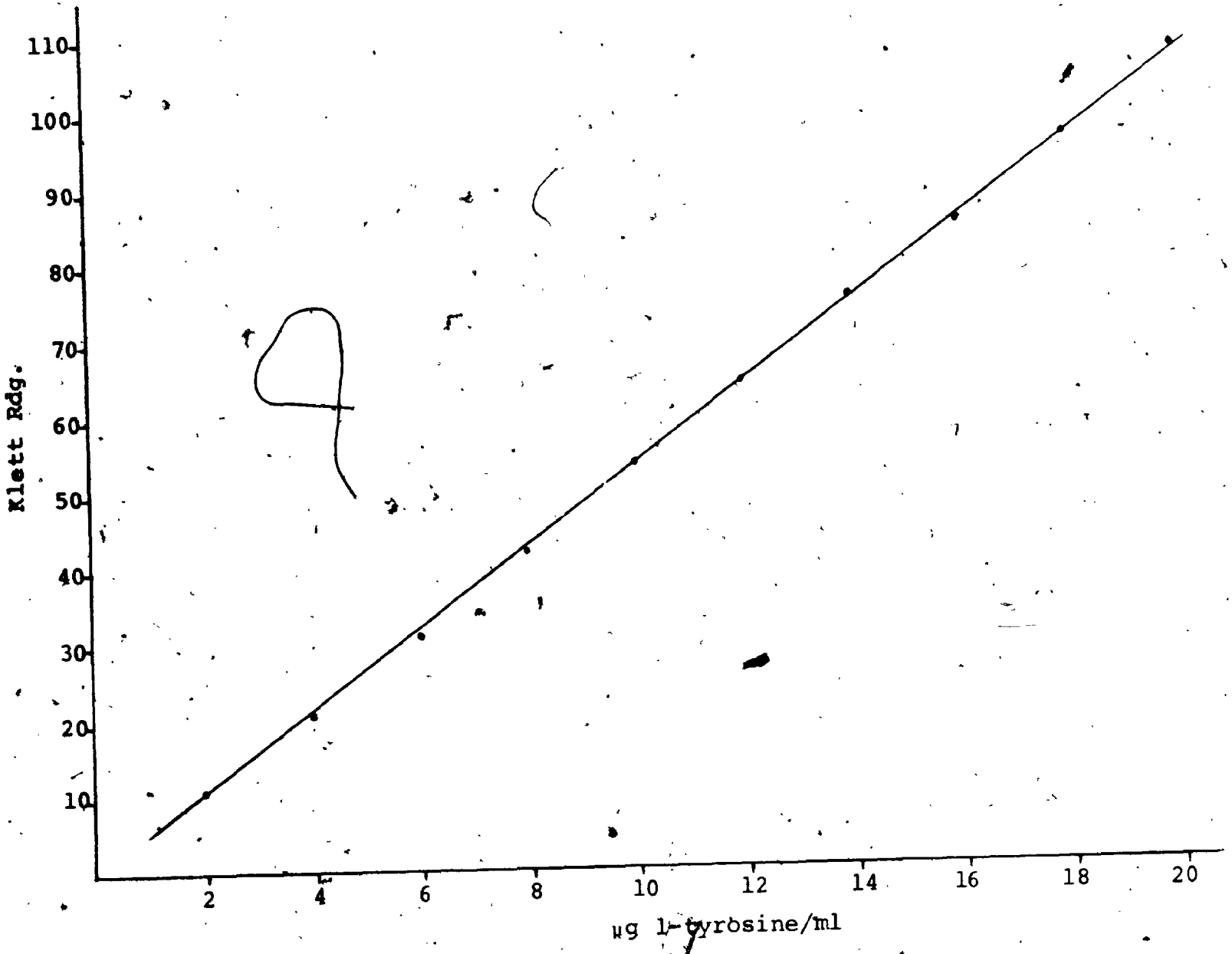


Figure 5. Standard curve: protease assay

3.3.2 Catalase activity

Principle: Catalase is reacted with excess hydrogen peroxide. The liberated oxygen is measured manometrically.

Reagents: (i) Phosphate buffer pH 6.5

3 g KH_2PO_4 and 2 g K_2HPO_4 were made up to 1 litre with distilled water.

(ii) Hydrogen peroxide substrate

Hydrogen peroxide (Fisher Scientific cat. No. H-325) was diluted with distilled water to give an 8-10% solution of peroxide.

Test procedure:

The assay of catalases and peroxidases was reviewed by Chance and Maehly (1955). It was reported that manometric methods are unacceptable for the accurate determination of catalase activity. However, it was emphasized that such techniques are useful and practical when using a catalase source of known activity. It was decided to use a manometric assay technique for catalase activity because it measures the evolution of oxygen under conditions similar to those used in the oxygenation studies reported in this thesis. The reaction of hydrogen peroxide and catalase was effected in the apparatus shown in Figure 6.

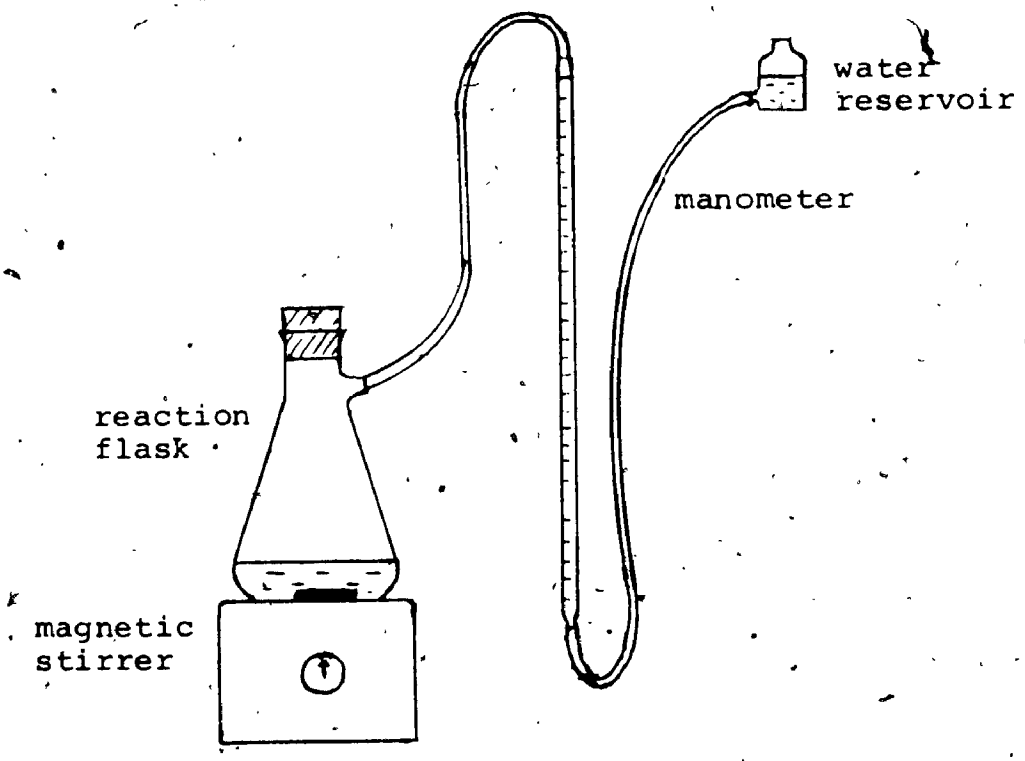


Figure 6: Apparatus for the determination of catalase activity

10 ml of the hydrogen peroxide solution (excess substrate) were added to 90 ml of phosphate buffer in a 250 ml suction flask. The water level in the 100 ml calibrated manometer tube was positioned to the zero mark by manipulating the sliding water reservoir connected to the base of the manometer. 1 ml of a suitably diluted catalase solution was added to the hydrogen peroxide, the flask was corked and the magnetic stirrer was activated to maximum speed. A gas volume equivalent to the oxygen liberated was collected in the manometer tube during a ten minute or other appropriate time interval. The water reservoir was continuously adjusted to maintain a pressure equivalent to atmospheric within the closed reaction and collection system. The test for catalase activity was carried out at ambient temperature, 25 ± 2°C. An error of 1.2% was associated with a temperature fluctuation of 1°C in the reaction flask.

3.3.3 Hydrogen peroxide determination

Principle: Hydrogen peroxide reacts with iodide in an acid medium to release iodine. The iodine is titrated with standard thiosulfate.

Reagents:
(i) KI solution, 10%
(ii) HCl, 1 N
(iii) ammonium molybdate, 3%
(iv) sodium thiosulfate, 0.2 N
(v) starch indicator

Test Procedure:

The general procedure outlined by Kolthoff and Sandell (1943) was followed. The hydrogen peroxide sample was appropriately diluted so that approximately 20 ml of standard thiosulfate would be required to titrate the liberated iodine. 2 ml of the KI solution, 2 ml of the HCl solution and 3 drops of ammonium molybdate were added to the hydrogen peroxide sample. The resultant iodine was titrated with standard thiosulfate to a starch indicator end-point.

3.3.4 Sodium sulfite

Principle: Excess standard iodine reacts to destroy all sulfite ions. The excess iodine is back-titrated with standard thiosulfate.

Reagents: (i) iodine solution, 0.3% I_2 in 2% KI

(ii) sodium thiosulfate, 0.02 N

(iii) starch indicator

Test Procedure:

Exactly 20 ml of the iodine solution were pipetted into the titration flask. An appropriate volume of sulfite solution was added and the excess iodine was titrated with standard thiosulfate to a starch indicator end-point. The sulfite ion concentration in the sample was calculated from the difference in the volume of thiosulfate required to titrate an iodine blank and the test sample.

3.3.5 Gluconic acid

Principle:

Gluconic acid as the product of the enzymatic oxidation of glucose is determined acidometrically. Excess sodium hydroxide is added to react with the gluconic acid and to stop further enzymatic action. The excess sodium hydroxide is back-titrated with standard hydrochloric acid.

Reagents: (i) NaOH, 0.10 N

(ii) HCl, 0.010 N

(iii) phenolphthalein indicator.

Test Procedure:

Exactly 2 ml of the NaOH solution were added to a 2 ml sample of the glucose - gluconic acid solution. The sample was back-titrated to a phenolphthalein end-point with the standard HCl solution. The difference in the volume of HCl required to titrate the sample at time zero and another sample at any given time was used as a direct index of the amount of gluconic acid that was present.

3.3.6 Dissolved oxygen measurements

A Beckman model 778 oxygen analyser was used to obtain dissolved oxygen concentrations in the stirred fermentations of Aspergillus. Since the polarographic oxygen probe responds to oxygen partial pressure, it was necessary to determine the solubility of oxygen in the fermentation medium at the temperature of fermentation in order to convert the partial pressure indication to dissolved oxygen concentration. The air-saturated solubility of oxygen in the fermentation medium at 31°C was found to be 6.5 ppm using the indigo-carmine method published by Jenkinson and Compton (1960). In this method reduced indigo-carmine dye reacts stoichiometrically with oxygen to produce an intense blue color that is measured by a colorimeter at 620 nm.

3.3.7 Biological solids determination

An estimate of the Aspergillus oryzae solids was obtained by filtering a measured volume of fermentor or shake flask contents, washing three times with an equivalent volume of distilled water and drying to constant weight at 85°C for 13-15 hours.

3.3.8 Viscosity

The viscosity of solutions was determined by a Brookfield syncro-lectric viscometer, model LVF, equipped with spindles appropriate for non-Newtonian fluids.

3.3.9 Agitation power

Power input to the fermentor contents was calculated from the read-out of the Baldwin Lima-Hamilton torque pick-up attached to the agitator shaft. The relationship between horsepower and torque is given by the following formula:

$$HP = \frac{T \times rpm}{63025}$$

where

HP = horsepower

T = torque in inch-pounds

rpm = revolutions/min

3.4 Microbiological Techniques

3.4.1 Preparation of sporulation agar

The Hasegawa sporulation medium (Table 5) was added in 50 ml aliquots to a series of 6 ounce medicine bottles each containing 1.5 g agar. The bottles were plugged with cotton wool and autoclaved at 120°C for twenty minutes. (Note: by avoiding a preliminary heat treatment to solubilize the agar, the medium set-up firmly). On removal from the autoclave, the contents of the bottles were swirled to ensure uniform distribution of the agar throughout the medium. The bottles were positioned at a 30° angle to the horizontal until the medium solidified, thereby exposing substantially more surface area for the development of Aspergillus spores.

3.4.2 Preparation of stock spore suspensions

The use of sterile distilled water to wash the Aspergillus spores from the surface culture on the agar slants proved to be unsatisfactory because the water failed to wet the conidia. An aqueous solution containing 0.1% Tween 80 (Atlas Powder Co.) and 0.1% sodium lauryl sulfate provided a practical answer to the problem. Approximately 20 ml of the above surfactant solution was layered over the spore mat in the medicine bottles and after a fifteen-minute "soaking" period, the bottles were shaken briskly by hand. A satisfactory, uniform spore suspension was obtained by this technique.

3.4.3 Preparation of spore cultures on agar slopes

Each medicine bottle containing 50 ml of sporulation agar was seeded with 1 ml of a spore suspension, prepared by the technique outlined above. The cultures were incubated at room temperature for a minimum of ten days to permit good spore development on the mycelial mat. Approximately one dozen bottles of sporulation agar were seeded at any one time. The resulting spore cultures were used as a source of spores for experimental fermentations and for subcultures to prepare fresh seed stock.

Fresh spore cultures were prepared as required. Spore cultures not used within a three-month period were normally discarded, though cultures six months old showed no apparent change in fermentation characteristics. One spore slant from each lot was set aside in cold storage (2°C) as a reference and control should retesting and culture examination be required.

3.4.4 Inoculation of experimental fermentations

The spore suspension in the 20 ml of surfactant solution was transferred from the sporulation bottle to 40 ml of sterile distilled water in a 300 ml conical flask. 2.5 ml of this diluted spore suspension were added to shake flasks containing 50 ml of sterile fermentation medium. For some of the more ambitious experiments, spores from several slants were pooled in a single spore suspension to ensure a uniform inoculum for

each shake flask. The sterile medium in the bench fermentor was inoculated with the spore equivalent of two culture slants. All fermentation media were sterilized in a steam autoclave at 120°C for twenty minutes.

CHAPTER 4: SHAKE FLASK STUDIES

Having selected a suitable culture of Aspergillus oryzae and a basic fermentation medium for protease production, it was of immediate interest to examine the response of the fermentation system to minor changes in media formulation and general fermentation conditions while, at the same time, providing an opportunity to become familiar with the physiological characteristics of the mycelia. Some basic information regarding the relative impact of pH, trace minerals and sugar concentration in the medium, as well as fermentation temperature and inoculation regimen on protease production was considered essential before embarking on any HPC studies.

The first few exploratory fermentations were carried out on a reciprocal shake table operating at 142 cycles/min in a temperature controlled room. When the New Brunswick incubator shake table became available, all subsequent fermentations were effected at 187 cycles/min and at temperatures usually in the 30-33°C range.

4.1 Trace Mineral Adjustment

The trace mineral content of the basic fermentation medium was initially as follows:

$ZnSO_4.7H_2O$	2 ppm
$FeSO_4.7H_2O$	20 ppm
$MnSO_4.H_2O$	20 ppm

The above levels of zinc, iron and manganese were arrived at somewhat arbitrarily by reviewing reports on published media formulations and using personal experience in industrial fermentations.

Preliminary shake flask fermentations, with and without trace minerals in the media, indicated the importance of these elements both for growth and protease production. The standard medium without the benefit of trace minerals led to restricted mycelial development and to zero protease titres at the end of an 86 hour cycle at 28°C. Though it seemed unlikely from the fermentation response that the initial trace mineral level was excessive, it was decided, somewhat arbitrarily, to reduce the iron and manganese concentrations in the standard medium to the following values:

$ZnSO_4.7H_2O$	2 ppm
$FeSO_4.7H_2O$	10 ppm
$MnSO_4.H_2O$	10 ppm .

4.2 Effect of Sulfate Ion

A paper published by Tomonaga et al. (1964) drew attention to the influence of sulfate ion in a defined medium on the production of protease by a specific strain of Aspergillus niger. In these reports the optimum concentration of sulfate ion for protease formation was 0.3×10^{-4}M, while for growth the optimum concentration was 1×10^{-3}M; the difference in concentrations being a factor of approximately 30.

More recently, Yanagita (1966, 1967), studying the kinetics of protease production with Aspergillus niger, showed that a sulfate deficient medium favored protease production as compared to a sulfate rich medium. The technique used required growth first in sulfate rich medium (1×10^{-3}M) followed by transfer to a sulfate deficient medium for protease production. The studies indicated that the apex of the hyphae determines the physiological age and that protease is secreted from a discrete region which is a fixed distance from the apex. This was deduced from measurements of the mean length of the hyphae as related to the time interval in the S+ medium and the time interval in the S- medium prior to the production of protease.

A calculation of the total sulfate concentration in the standard medium selected for the Aspergillus oryzae indicated a value of 105×10^{-4}M, of which 104×10^{-4}M originated from the $MgSO_4$ and 1×10^{-4}M originated from the trace minerals that were present. The sulfate ion concentration was an order of

magnitude higher than the levels reported above as optimum for protease production by Aspergillus niger and prompted an examination of the protease response of the Aspergillus oryzae culture over a wide range of sulfate concentrations. Two sets of shake flask fermentations were carried out with sulfate concentration that ranged 100 fold. The magnesium ion concentration was kept constant by adjusting, as required, with $MgCl_2.6H_2O$.

4.2.1 Effect of sulfate on protease synthesis

In this fermentation study the total amount of magnesium sulfate was replaced with an equivalent amount of magnesium chloride and the sulfate concentration in the flasks was adjusted with sodium sulfate. The initial pH of each flask was 5.4 and the fermentation temperature was 28°C. The shake table was inadvertently stopped for eighteen hours sometime during the 89-113 hour interval. The results are shown in Table 7 below.

Table 7:

Effect of sulfate ion on protease titre

Flask No.	Sulfate conc. 10^{-4} M	Ferment. time, hours	pH	Protease titre, mU/ml
1	1	89	5.0	18
2	1	89	4.8	18
3	1	89	4.6	12
4	1	113	4.9	85
5	1	113	5.2	102
6	1	113	5.1	89
7	4.5	89	4.8	24
8	4.5	89	4.8	17
9	4.5	89	4.8	20
10	4.5	113	5.5	72
11	4.5	113	5.9	148
12	4.5	113	5.2	89
13	11.5	89	4.8	12
14	11.5	89	4.8	15
15	11.5	89	4.7	11
16	11.5	113	5.4	43
17	11.5	113	5.2	33
18	11.5	113	5.2	33

4.2.2 Sulfate and protease synthesis

The shake flask fermentations in this set were carried out in standard fermentation medium in which the magnesium sulfate was replaced as required with magnesium chloride to maintain the original magnesium ion concentration. The initial pH of the flasks was 5.3 and the fermentation temperature was 28°C. The results are recorded in Table 8.

Table 8:

Effect of sulfate ion on protease titre

Flask No.	Sulfate conc. 10^{-4}M	Ferment. time, hours	pH	Protease titre, mU/ml
1	1	96	4.8	26
2	1	96	5.1	23
3	1	108	5.0	31
4	1	108	5.0	25
5	3	96	5.1	50
6	3	96	5.6	34
7	3	108	5.4	98
8	3	108	5.6	97
9	105	96	6.4	170
10	105	96	6.4	161
11	105	108	6.7	47
12	105	108	6.6	78

Flasks with the lowest sulfate concentration in both sets of fermentations yielded appreciably less mycelial mass than the remaining flasks. Unfortunately, the pattern of protease production was obscured by the seemingly random development of pellet and filamentous growth in sister flasks. In general, the higher sulfate level of the basic medium appeared to favor filamentous growth without militating against the production of protease; consequently it was decided to maintain the sulfate level at 105×10^{-4}M, as originally selected, and further investigations in this direction were discontinued.

4.3 Effect of pH

The pH of biological media is known to be a most important parameter. Each organism has its optimum pH for growth and for product formation. In many fermentations the optimum pH for growth does not coincide with the optimum pH for product formation, consequently close control of the pH during the fermentation cycle may be necessary to optimize the yield. It was considered imperative to check the response of the Aspergillus oryzae culture in media that had been adjusted over a selected pH range.

Four portions of the standard fermentation medium were adjusted with HCl or NH_4OH, as required, to give four sets of shake flask fermentations that covered the pH range from 4-6. The fermentation temperature was 28°C. The protease titres at different fermentation times for each set of flasks are shown in Table 9.

Table 9:

Effect of initial media pH on protease titres

Flask No.	Initial pH	pH			Protease titre, mU/ml
		70 h	89 h	108 h	
1	3.9	3.3			7
2	3.9	3.3			7
3	3.9		3.6		14
4	3.9		3.6		12
5	3.9			3.9	20
6	3.9			3.9	70
7	4.6	3.9			12
8	4.6	3.9			9
9	4.6		6.2		56
10	4.6		4.6		14
11	4.6			4.7	24
12	4.6			6.0	102
13	5.4	4.0			11
14	5.4	4.0			11
15	5.4		6.2		62
16	5.4		4.4		14
17	5.4			5.1	41
18	5.4			5.0	27
19	6.0	4.4			8
20	6.0	4.4			7
21	6.0		5.2		20
22	6.0		5.1		20
23	6.0			5.7	80

The fermentation pattern indicated an initial drop in pH during the first three days of cell growth, followed by a steady increase in pH coincident with the accumulation of protease in the fermentation broth. It was apparent that considerable latitude was permissible in the initial pH of the fermentation medium. Random differences in the morphological characteristics, i.e. pellet vs. filamentous growth, of the mold were implicated in the disparity of the protease titres that were measured. On the basis of the results reported above, the originally selected pH of 5.4 for the standard medium was accepted without change.

4.4 Effect of Increased Sugar Concentration

As a general rule, each gram of sugar that is metabolized by a mold culture during the growth phase will produce approximately one half gram of dry mycelial solids. The standard fermentation medium adapted from Tosoni and Glass (1963) contained a total of 1.09% carbohydrate and it was felt that mycelial solids determinable in the 0.3-0.5% range were marginally low for the prospective oxygen transfer studies in stirred fermentations.

The sugar concentrations in the basic fermentation medium were doubled to provide for additional biomass and correspondingly higher protease titres. Shake flask fermentations at 28°C demonstrated an increase in protease titres from a high of 216 mU/ml in the basic medium to a high of 467 mU/ml in the fortified medium. The increase in sugar concentration

effectively doubled the mycelial solids and the protease titres that were formerly encountered. The fermentation cycle, however, was extended an additional 24-36 hours to approximately six days.

On the basis of the above response, the sugar concentrations in the fermentation medium were adjusted to 1.5% sucrose and 0.75% glucose. Subsequent fermentations were effected at this carbohydrate concentration.

4.5 Distilled, De-ionized and Tap Water in Media Preparation

The use of tap water to replace distilled water was examined as a convenient alternative in the preparation of the standard medium, particularly when relatively large volumes would be required for the investigations in the stirred-fermentor.

Three sets of shake flask fermentations were undertaken to assess the effect of tap water on the final protease titre. The first two sets were fermented at 28°C on the shake table operating at 142 cycles/min and the remaining set was fermented at 33°C on the incubator shake table operating at 187 cycles/min. The results of these fermentations are tabulated below in Tables 10, 11, and 12.

Table 10:

Fermentation response of Aspergillus oryzae in media prepared from distilled water and London well water

Source of water	Flask No.	Ferment. time, hours	pH	Protease titre, mU/ml
distilled	1	117	6.82	262
	2	117	6.80	379
	3	159	6.75	321
	4	159	6.60	266
London well	5	117	6.85	27
	6	117	5.40	4
	7	159	6.65	62
	8	159	6.75	70

Table 11:

Fermentation response of Aspergillus oryzae in media prepared from distilled water and a blend of Lake Huron and London well water

Source of water	Flask No.	Ferment. time hours	pH	Protease titre mU/ml
distilled	1	130	5.00	20*
	2	130	6.65	313
	3**	--	-	-
	4	155	6.70	322
unknown blend of London well and Lake Huron	5	130	5.15	8*
	6	130	6.65	29
	7	155	5.60	70
	8	155	6.80	53

* pellet formation of mycelia

** flask broke

2 3

OF/DE

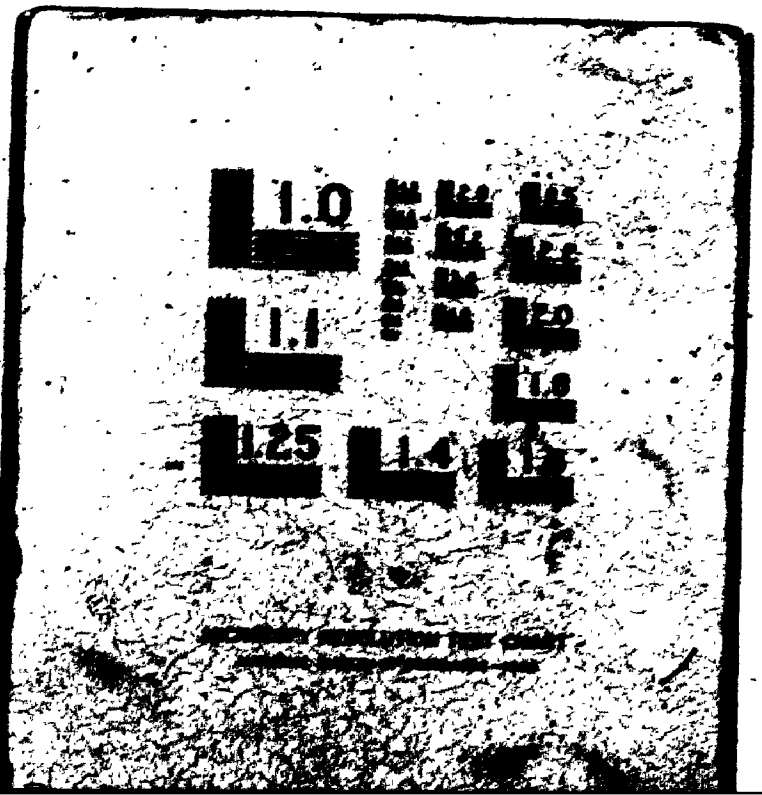


Table 12:

Fermentation response of Aspergillus oryzae in media prepared from distilled, de-ionized and Lake Huron waters

Source of water	Flask No.	Ferment. time, hours	pH	Protease titre, mU/ml
distilled	1	106	6.80	289
	2	106	6.90	258
	3	106	6.85	254
	4	106	6.90	264
	5	106	6.65	369
	6	106	6.70	359
	7	106	6.85	406
	8	106	6.70	443
				330 avg.
de-ionized	9	106	6.85	320
	10	106	6.80	375
	11	106	6.85	406
	12	106	6.80	324
	13	106	6.60	418
	14	106	6.60	418
	15	106	6.80	451
	16	106	6.70	553
				406 avg.
Lake Huron	17	106	6.50	100
	18	106	6.70	131
	19	106	6.55	68*
	20	106	6.45	78*
	21	106	6.70	160
	22	106	6.65	219
	23	106	6.65	217
	24	106	6.70	174
				143 avg.

* pellet formation of mycelia

It was clear from the above results that London tap water from Lake Huron could not be used in media formulation. No attempt was made to determine the precise nature of the toxic agent in the local tap water, but it was presumed that the fluoride content (1 ppm) was the most likely inhibitor. Henceforth all media were standardized on the use of de-ionized water which was somewhat more readily obtainable than distilled water and also produced the highest protease titres.

4.6. Selection of Fermentation Temperature

The initial shake flask fermentations were carried out on an open shake table that was placed in a temperature controlled room. It was not possible, due to the constraints of the heating system for the room, to explore temperatures higher than 28°C until such time as an incubator shake table was made available. Though the optimum fermentation temperature for the selected strain of Aspergillus oryzae was not determined, the range of 31-33°C, except as noted, was used for the remaining experimental fermentations report here. This higher temperature range resulted in improved protease titres in a shorter time than at 28°C. Fermentation temperatures higher than 33°C were not examined because that temperature was the practical upper limit of the temperature control system of the stirred bench fermentor.

4.7 Selection of Antifoam

Most stirred aerobic fermentations require the addition of small quantities of an antifoam agent to control foam levels in the head space of the fermentor. A good antifoam agent, in addition to its surface active properties, must be non-toxic to the organism, it should not interfere with the recovery of the fermentation product and preferably it should be metabolized by the culture in such manner as to enhance the yield of product. The production of protease by Aspergillus oryzae in the presence of different antifoam agents was examined in shake flask fermentations. The results of several fermentation sets are presented in Tables 13 and 14.

Table 13:

The effect of antifoam agents on the production of protease by *Aspergillus oryzae*

Antifoam treatment	Ferment. time, hours at 28°C	Protease titre, mU/ml
controls	159	240, 290
silicone DC 3* @ 80 ppm	159	n/a*** 22
silicone DC 3 @ 120 ppm	159	n/a 0
controls	115	211, 224
lard oil @ 0.4%	115	278, 217
lard oil @ 2%	115	252, 204
olive oil @ 0.4%	115	224, 285
olive oil @ 2%	115	137, 204
light paraffin oil @ 0.4%	115	132, 105
light paraffin oil @ 2%	115	36, 34
control	155	n/a 322
oleic acid @ 0.4%	155	n/a 127
oleic acid @ 2%	155	n/a 47
control	116	n/a 226
Hodag** KG-1 @ 2%	116	n/a 8
Hodag M-8 @ 2%	116	n/a 295
Hodag M-9 @ 2%	116	n/a 100
Hodag M-10 @ 2%	116	n/a 69

* Dow Corning Co., Toronto

** Hodag Chemical Corp., Chicago

*** not available

Table 14:

The effect of Hodag M-8 on the production of protease by Aspergillus oryzae

Antifoam treatment	Flask No.	Ferment. time, hours at 33°C	pH	Protease titre, mU/ml
controls	1	62	6.30	221
	2	86	6.55	455
	3	110	6.75	500
	4	134	6.70	545
Hodag M-8 @ 2%	5	62	5.50	12
	6	86	5.65	111
	7	110	6.30	820
	8	134	6.00	672

Hodag M-8, a proprietary fatty acid mixture, was found to be the best of the various antifoam agents that were examined. It was obvious that the silicone, paraffin oil, oleic acid and three of the Hodag agents were toxic to varying degrees at the concentrations used in the tests and no additional fermentations were carried out with these materials. The Hodag M-8 was obviously metabolized by the Aspergillus oryzae as evidenced by a delay of at least 24 hours before the onset of protease production and a peak titre significantly higher than the controls. Hodag M-8 was clearly the antifoam agent of choice and was added at a concentration of 0.05% to all media in the stirred bench fermentor.

4.8 Investigation of Protease Induction

The induction of enzyme secretion by the addition of specific substrate materials to fermentation media is well established. It has been shown (Tanabe and Tonomura, 1954; Tonomura et al., 1961) that α-amylase formation by Aspergillus oryzae is induced by the addition of starch, glycogen, maltose and isomaltose, but not by the addition of glucose, which, in fact, inhibits α-amylase formation. Terui (1968) reported that peptone and casein added to a culture of Aspergillus niger, in the growing phase, stimulated protease formation in the subsequent non-growth phase of the culture.

To capitalize on an inductive effect to increase the yield of protease, gelatin and peptonized milk were added to the standard medium for assessment in shake flask fermentation. The results of a single exploratory fermentation at 33°C are shown in Table 15 below.

Table 15:

The effect of gelatin and peptonized milk on the production of protease by Aspergillus oryzae

Protease inducer	Ferment. time, hours	pH	Protease titre, mU/ml	
controls	95	6.70	468,	505
gelatin, 2%	95	8.10	n/a*	49
Bacto peptonized milk 0.15%	95	7.05	n/a	455

* not available

The results did not indicate any inductive response. The relatively high pH of the gelatin fermentation was most likely responsible for the low protease titre that was registered.

4.9. Effect of Seed Inoculum: Spores versus Vegetative Cells

The use of vegetative mycelia was examined as a possible replacement of the spore inoculum for future fermentations. Inocula of spores that had been stored 3.5 months at room temperature and mycelia that had been stored three months at 1°C were compared for protease production in the standard fermentation medium. This shake flask experiment was carried out at 28°C and the results are reported in Table 16 below.

Table 16:

The production of protease by Aspergillus oryzae as a response to inocula at spores and mycelia

Inoculum	Flask No.	Ferment. time, hours	pH	Protease titre, mU/ml
spore suspension 2.5 ml	1	113	4.60	22
	2	113	6.55	277
	3	113	6.50	268
	4	113	6.60	308
	5	137	6.70	373
	6	137	6.75	467
vegetative mycelia 3 ml	7	113	3.60	0
	8	113	3.65	0
	9	113	3.65	0
	10	113	3.65	0
	11	137	3.65	0
	12	137	3.65	15

The flasks seeded with vegetative cells produced a creamy-white lumpy mass of mycelia and typically low pH values. Concurrently with the smaller 50 ml shake flask tests, a pair of 2 litre shake flasks, each containing a litre of fermentation medium, was inoculated with 15 ml of vegetative cells and 10 ml of spore suspension, respectively and placed on the shaker. Both flasks produced filamentous mycelia. The spore-seeded flask produced 308 mU/ml of protease after 137 hours, whereas the

mycelia-seeded flask produced a mere 56 mU/ml of protease in the same period. The pH of this latter flask reached a more respectable 6.25.

Though the mycelia-inoculum in the above experiment was stored very much longer than would be considered prudent for good culture management, the implication was clear; spores offer much greater culture-stability than the corresponding vegetative form. The spores stored for 3.5 months at room temperature did not demonstrate any apparent loss of viability or of latent potential for protease productivity.

4.10 Physical Factors Relating to Pellet Formation

It had become increasingly apparent that poor protease titres could be expected from shake flasks in which the mold developed initially as pellets and subsequently agglomerated to a lumpy mass. Flasks with filamentous growth, which appeared slushy and pulpy, generally gave significantly higher enzyme titres than flasks with lumpy biomass.

The reason for pellet formation, as opposed to filamentous formation, was not clear. The random nature of the occurrence of the two forms of growth suggested that complex reactions were involved. Ohama et al. (1966) reported that in specific instances, when low numbers of Aspergillus niger spores were used as a seed inoculum, pellet formation was encountered with concomitant low protease titres. However

when larger numbers of spores were used, the hyphae grew in a pulpy manner with correspondingly higher mycelial mass and protease production. Fukimbara (1966) noting that "pellet formation depends upon mainly physical and mechanical factors", demonstrated that short sterilizing times (two minutes) of culture media rather than long sterilizing times (sixty minutes) favored the development of filamentous growth and production of α-amylase by Aspergillus awamori.

A factorial experiment was carried out to test the morphological and protease response of the Aspergillus oryzae culture to discrete changes in relevant physical factors. Three factors were investigated: sterilization time at two levels, seed inoculation size at two levels, and inert solids at three levels. Half the medium was autoclaved for three minutes at 120°C and the other half was autoclaved for thirty minutes at 120°C. The spore inoculum was adjusted to 1 ml of standard spore suspension for half the flasks and 5 ml for the other half. An inert cellulose powder, Alpha-floc*, was added to an appropriate number of flasks at 0, 390 and 1800 ppm concentrations. These shake flask fermentations were carried out at 33°C in the incubator shake table. The assay results in duplicate are given in Table 17.

The mycelial growth in all but two of the shake flasks was well dispersed and floccose in appearance. The characteristically low protease titres in the two flasks with lumpy biomass precluded a rigorous analysis of the data by statistical methods. To permit a qualified statistical inference, the average protease assay for each set of seven flasks containing 390 and 1800 ppm Alpha-floc, respectively, was substituted for the abnormally low assay.

*General Refractories Co., Berlin, New Hampshire.

Table 17:

The production of protease (mU/ml) by Aspergillus oryzae in response to changes in fermentation parameters

A. oryzae	Alpha-floc, 0 ppm		Alpha-floc, 390 ppm		Alpha-floc, 1800 ppm	
	Medium autoclaved 3 min	Medium autoclaved 30 min	Medium autoclaved 3 min	Medium autoclaved 30 min	Medium autoclaved 3 min	Medium autoclaved 30 min
spore inoculum, 1 ml	287	480	492	472	82*	451
	475	492	41*	451	427	414
spore inoculum, 5 ml	398	459	443	410	336	328
	373	451	328	398	369	303

*These flasks contained lumpy biomass.

The matrix of protease assays was examined by analysis of variance (Appendix 1). The statistical analysis indicated that the lower inoculation rate improved the protease titres (5% level of significance). There were no significant interactions. The protease assays from the separate treatments were sufficiently close that no change in the established fermentation procedures, outlined in Section 3.4, was warranted.

4.11 The Effect of Polymers on Mold Morphology

The random occurrence of pellet and lumpy mold formation in the early shake flask fermentations, together with their generally poor enzyme titres, made it very difficult to draw useful inferences from many of the results. Both pellet and filamentous growth frequently appeared in different flasks within a set of replicate fermentations and, indeed, both forms sometimes appeared in the same flask. Whatever the reason or reasons for this anomalous fermentation behaviour, it was obvious from the results of many shake flask experiments that the problem would not lend itself to a simple solution.

The addition of macromolecules to fermentation media has been shown to improve product yield in certain instances. Botri et al. (1964) claimed that the introduction of polyvinyl alcohol, M.W. 30,000, to penicillin broths at a concentration

of 40 ppm, increased the yield of penicillin by 38%. Other macromolecules such as polyvinyl pyrrolidone and sodium polyacrylate gave similar but less dramatic responses. Corman (1959) claimed an increase in the yield of β-carotene from 22 gamma/ml to 326 gamma/ml when the apparent viscosity of the fermentation broth was increased to 600 cps by the addition of thickening agents such as starch or carboxymethyl cellulose. The increase in yield was thought to be associated with the dispersed growth of the Blakeslea trispora in the thickened broth.

The above observations suggested a possible technique for obtaining uniform filamentous growth in the Aspergillus oryzae culture under investigation. Carbopol 941 (a carboxy vinyl polymer selected at random from several available in the laboratory) was added to the standard fermentation medium and the response was compared with appropriate control flasks for mold morphology and protease titres. The results were dramatic to say the least. At 60 hours the mold in the shake flasks treated with Carbopol at 1000 ppm was uniformly dispersed in the medium and well ahead of the control flasks in the elaboration of biomass. At 96 hours the Carbopol flasks contained slushy mycelial mass and assayed at twice the protease levels found in the controls, which showed a marked tendency for a pellet form of mycelial development.

Though the initial protease titres obtained with Carbopol were by no means the highest observed from shake flasks without benefit of added polymer, the rapid development of the filamentous form was sufficiently exciting to initiate a survey of assorted polymers on hand in the laboratory. Table 18 combines the results of three separate series of shake flask fermentations in this area.

Table 18:

The morphological and protease response of Aspergillus oryzae in the standard fermentation medium supplemented with commercially available polymers

Polymer*	Use conc. ppm	Final pH	Protease titre, mU/ml	Growth form	Ferment. time/temp.
control	-	5.20	31	pellet	
control	-	5.75	47	pellet	
Polyclar H	1000	5.65	37	pellet	
Polyclar H	1000	6.00	74	pellet	108 h
PVP 10,000	1000	5.35	12	pellet	at
PVP 10,000	1000	5.40	0	pellet	28°C
NaCMC	1000	6.55	78	pellet	
NaCMC	1000	6.50	164	pellet	
Carbopol 940	1000	6.55	201	filamentous	
Carbopol 940	1000	6.30	201	filamentous	
Carbopol 941	1000	6.55	221	filamentous	
Carbopol 941	1000	6.55	211	filamentous	
control	-	4.00	9	pellet	
Carbopol 940	1000	6.40	172	filamentous	
Carbopol 941	1000	6.40	153	filamentous	
Carbopol 960	1000	6.35	142	filamentous	
Carboxel X-385	1000	6.15	57	filamentous	106 h
Separan NP 10	1000	5.65	34	filamentous	at
Methocell MC 4000	1000	5.70	39	filamentous	23°C for
Polymer 705 DA	1000	6.20	77	filamentous	first 50 h
Jaquar J251	1000	6.30	111	filamentous	remainder
Methofas M4500	1000	5.90	40	filamentous	at 28°C
Methofas MPM	1000	5.20	16	filamentous	
control	-	4.00	0	pellet	
control	-	4.20	0	pellet	
Carbopol 940	100	6.40	111	filamentous	109 h
Carbopol 940	100	6.60	174	filamentous	daily
Carbopol 940	200	6.50	109	filamentous	temp.
Carbopol 940	200	6.55	160	filamentous	range
Polyox WSR-301	50	5.90	0	pellet and filamentous	23-27°C
Reten A-1	50	3.90	0	pellet	

Polyclar H and PVP 10,000:	polyvinylpyrrolidone type manufactured by Antara Chemicals, New York, N.Y.
NaCMC:	sodium carboxy methyl cellulose manufactured by Canadian Industries Ltd., Montreal, P.Q.
Carbopols:	carboxy vinyl polymers manufactured by B.F. Goodrich Ltd., Kitchener, Ont.
Carboxel X-385:	modified cellulose type manufactured by Chemical Developments of Canada Ltd., Montreal, P.Q.
Separan NP 10:	polyacrylamide type manufactured by Dow Chemical of Canada Ltd., Sarnia, Ont.
Methocel MC 4000:	modified cellulose type manufactured by Dow Chemical of Canada Ltd., Sarnia, Ont.
Polymer 705 DA and Jaquar J 251:	polyglycosides extracted from guar by Stein-Hall & Co. Ltd., New York, N.Y.
Methofas M4500 and Methofas MPM:	modified celluloses manufactured by Canadian Industries Ltd., Montreal, P.Q.
Polyox WSR-301:	polyethylene oxide type manufactured by Union Carbide Ltd., Toronto, Ont.
Reten A-1:	polyacrylate type manufactured by Hercules Inc., Wilmington, Del.

The inference from the results of the above shake flask fermentations was that media containing Carbopols promoted rapid development of a uniformly dispersed filamentous mycelial mass and produced higher protease titres than the controls and the treatments with the other polymeric types. Unfortunately, the low protease titres in the control flasks were compromised by the pellet growth characteristics of the mold.

To establish the optimum use concentration of Carbopol for protease production, shake flask fermentations were carried out in the presence of a wide range of polymer concentrations in the fermentation medium. The results obtained after 88 hours on the shake table at 25°C are noted in Table 19 below.

Table 19:

Protease response of Aspergillus oryzae to a wide range of Carbopol concentrations in the fermentation medium

Conc. of Carbopol 940 in media, ppm	Final pH	Protease titre, mU/ml
0	3.80	0
40	6.00	4
80	6.30	100
160	6.25	68
280	6.25	98
500	6.10	47
1000	5.40	49
2000	6.00	0

It appeared that the optimum concentration of Carbopol was in the 80 to 280 ppm range; considerably less than the 1000 ppm concentration originally used. A subsequent shake flask fermentation series examining sugar concentrations in the fermentation media supported the above contention. The fermentation cycle was 109 hours on the shake table at 27°C and the results are recorded in Table 20.

Table 20:

Fermentation response of Aspergillus oryzae to variations in Carbopol and sugar concentrations in the fermentation media

Sugar level	Carbopol 940, ppm	Protease titre, mU/ml	Growth form
media without sugar	0	0	no visible growth
	20	25	no visible growth
	200	16	no visible growth
	1000	6	no visible growth
media with half normal sugar level	0	162	pellet
	20	144	filamentous
	200	125	filamentous
	1000	106	filamentous
media with normal sugar level	0	-	(flask broke)
	20	164	filamentous
	200	256	filamentous
	1000	200	filamentous

Carbopol at a concentration of 200 ppm was found to promote good protease yields. The control flask, in the set with half the normal sugar level, produced an unusually high protease titre in the presence of a pellet growth form of the mold. The mycelia in the remaining flasks of the set appeared to have undergone appreciable autolysis after the carbohydrate source

was exhausted and this was reflected in the somewhat reduced protease titres that were found. It was also clear that Carbopol does not support the growth of Aspergillus oryzae. The small but positive protease titres that were obtained in the set without fermentable carbohydrate, were likely due to some interaction of the polymer on the surface of the spores.

At first it was thought that the Carbopols promoted filamentous growth of the mold in response to an increase in the viscosity of the fermentation medium. Exploratory fermentations on the shake table indicated that Carbopol 940 and Carbopol 941 could be used interchangeably without compromising the protease titres or morphological characteristics of the mold. This, of course, is not surprising since the chemical and physical properties of both polymers are very similar.

Bench tests were carried out with Carbopol to establish pH, concentration, shear and temperature effects on viscosity. Carbopol dissolved in distilled water and neutralized with either caustic soda or ammonium hydroxide, possesses a pseudo-plastic rheological behaviour. For example, the apparent viscosity of a 0.1% solution of Carbopol 941 in distilled water was determined (see Table 21) before and after neutralization with NH_4OH.

Table 21:

Apparent viscosities of a 0.1% solution of Carbopol 941

Temp. °C	pH	Spindle speed, rpm	Spindle #	Apparent viscosity, cps
27	3.55	60	2	116
27	6.80	6	3	8600
		12	3	5250
		30	3	2720
		60	3	1690
35	6.85	6	3	7940
		12	3	4800
		30	3	2500
		60	3	1550

From the above rheological data, the flow behaviour index 'n' and the consistency index 'K' of the 0.1% Carbopol solution at 27°C were calculated to be 0.3 and 209, respectively. Variations in apparent viscosity due to temperature fluctuations within the normal fermentation range (27-35°C) would appear to be minimal. Furthermore, the Carbopol polymers are reasonably stable to the application of heat. It was determined that approximately 95% of the apparent viscosity of a 0.1% solution of Carbopol was

recovered after thirty minutes in the autoclave at 122°C. There was, however, the possibility that the polymer could undergo rapid mechanical degradation in the shear zone created by the impeller of a stirred fermentation. To examine the effect of prolonged stirring on the apparent viscosity, 1 litre of a 0.1% solution of Carbopol 941 (neutralized) was stirred in a 14-litre fermentor jar for 90 hours. A single turbine impeller at an agitation rate of 190 rpm was used. Samples were taken at appropriate intervals and the apparent viscosity was measured. Table 22 below shows the values that were obtained.

Table 22:

The effect of prolonged stirring on the apparent viscosity of a 0.1% solution of Carbopol 941

Spindle speed, rpm	Apparent viscosity, cps				
	Stirring time, hours				
	0	18	39	68	90
6	7900	6300	5500	5300	4900
12	4800	3870	3420	3250	3070
30	2510	2080	1850	1800	1700
60	1575	1325	1180	1050	1080

The progressive reduction in apparent viscosity over the 90 hour stirring cycle indicated that appreciable mechanical destruction of the polymer molecules could be expected in prospective fermentations. The results, nevertheless, suggested that sufficient integrity of the polymer would persist during the critical growth phase of the mold.

Though Carbopol added to the standard fermentation medium in the 200-1000 ppm range did not appear to markedly increase the viscosity of the mixture, it was assumed that the pH of the medium, being somewhat on the acid side, accounted for this effect. There was the possibility that polymer molecules adsorbed onto the mycelial surface might undergo a marked viscosity increase within a potentially more neutral pH environment at the cell - liquid interface. However, viscosity measurements of the standard fermentation medium at pH 7 containing 1000 ppm Carbopol established the fact that the viscous properties of the polymer were effectively destroyed by one or more of the ingredients in the medium. (Table 23). The apparent viscosity readings were taken at a spindle speed of 60 rpm and temperature 27°C.

Table 23:

The effect of the standard fermentation medium on the viscous properties of Carbopol

Treatment	pH	Apparent viscosity, cps
control	5.35	2.1
	7.0	2.1
medium + 1000 ppm Carbopol 940	4.80	2.1
	7.0	2.5
medium + 1000 ppm Carbopol 941	4.75	2.7
	7.0	3.0
medium + 1000 ppm Carbopol 960	5.45	2.3
	7.0	2.3
distilled water + 1000 ppm Carbopol 940	3.70	4.0
	6.1	990
distilled water + 1000 ppm Carbopol 941	3.55	116
	6.8	1690

Tests with the individual components of the standard medium indicated that neutralized lactic acid and KH_2PO_4 completely destroyed the viscous properties of the Carbopols. The trace minerals present in the medium also contributed to the breakdown of the polymer viscosity, but to a lesser

extent than the lactic acid and phosphate. The sugars supported the rheological integrity of the Carbopol polymers and, in fact, induced a 30% increase in apparent viscosity. It seemed highly improbable that the effect of Carbopols on the fermentation behaviour of Aspergillus oryzae was attributable to any rheological properties of the polymer. The observations would suggest that the Carboppl molecules can exist in two physical states, globular and fibrous, the latter state imparting the marked pseudoplastic flow behaviour of neutralized solutions. Presumably, a salt, or other effect, induces a conformational change in the polymer molecules and causes a rapid transition from the fibrous to the globular form with concomitant destruction of gel properties.

This still leaves a question as to how Carbopol promotes filamentous growth.

In an extension of the research reported above, Moo-Young* et al. (1973a, 1973b) examined the effect of Carbopols and other polymers on mass transfer rates in Aspergillus niger fermentations. It was noted that the Carbopol polymers were not degraded by the culture and that the mycelia were well

*The author of this thesis worked under the direction of Dr. Moo-Young during his tenure at the University of Western Ontario prior to accepting an appointment at the University of Waterloo.

dispersed and pulpy in consistency. Results showed significant increases in the rate of amylase production, maximum growth rate, CO_2 evolution and glucose uptake. The enhancement effects could not be correlated to the rheological properties of the Carbopol and were ascribed largely to an increased interfacial area for nutrient transfer. Experiments with mold pellets in the presence of Carbopol demonstrated an increase in potassium transport across the cell membrane which was suggested as an explanation for some of the enhancement effects. Speculation was also made that hydrodynamic "slip", induced by a film of Carbopol at the pellet interface with the liquid menstrum, might potentiate mass transfer.

Since it was established that Carbopols influenced the growth pattern of Aspergillus oryzae for reasons other than their rheological properties, further tests were not required. For all subsequent shake flask fermentations, Carbopol 940/941 at 200 ppm was added to the culture media to promote the development of filamentous mycelia.

CHAPTER 5: SULFITE OXIDATION RATES

5.1 Fermentor Aeration Studies

The sulfite method for measuring the oxygen absorption coefficient in a stirred reactor, was introduced by Cooper, Fernstrom and Miller (1944) in their classic paper. Though the method may be criticized because it does not measure oxygen transport under in situ fermentation conditions, it does provide a realistic basis for comparison of oxygen transport rates under specific stirring regimes as well as establishing a maximum transfer rate expectancy within a defined set of operating conditions. The main objective of this study was to demonstrate the relative reduction in oxygen transport when a Newtonian system is compared to a non-Newtonian system and to investigate the application of the technique for supplying oxygen to fungal fermentations.

Oxygen absorption rates were determined in the 14 litre bench fermentor under a variety of aeration rates, agitation levels, and solids suspension concentrations that might be encountered under actual fermentation conditions. 36 g of Na_2SO_3 were added to 12 litres of water at 22-24°C in the fermentor. Copper sulfate (1×10^{-4} M) was added as an oxidation catalyst. The reduction in sulfite concentration per unit time was determined iodimetrically and reported as the sulfite oxidation rate (SOR) in mM O_2/litre-min.

The air flowmeter and stirrer tachometer which are standard equipment on the bench fermentor were not sufficiently accurate to permit a definitive study of sulfite oxidation rates; however it was considered useful to establish the approximate decrease in oxygen transport in the presence of paper pulp*, cellulose powder (Alpha-floc), antifoam and carboxy methyl cellulose (Carboxel X-385). As mentioned in the literature review, Brierley and Steel (1959) demonstrated that paper pulp could be used satisfactorily in lieu of the mycelia of Aspergillus niger for oxygen absorption studies. A 90% reduction in the oxygen absorption rate was effected by a 2% (dry weight) suspension of mycelia, and an 85% in oxygen absorption rate was effected by a 2% (dry weight) suspension of paper pulp. Gaden (1956) in similar studies showed that 1% cell tissue reduced the oxygen transport coefficient by 80%.

One of the adverse effects of antifoam agents, as used in fermentations, is to lower the oxygen transport coefficient. Phillips et al. (1960) and Solomons and Perkins (1958) presented data to show that typical antifoam agents such as silicones, lard oils, etc. can be expected to reduce oxygen transport by about 50%. Chain and Gualandi (1966) have published data on the effect of antifoam in 3000 litre fermentations. The antifoam caused the dissolved oxygen concentration in the menstrum to drop from about 50% of

*Paper pulp was obtained by shredding 20 g quantities of filter paper with water in a Warring-type blender for thirty seconds.

saturation to zero, indicating that the demand of the organism could no longer be satisfied.

The results of the sulfite oxidation studies are reported in Tables 24, 25, 26, 27, 28 and 29 which follow.

The results of the sulfite oxidation tests were generally consistent with observations reported by Brierley (1959), Maxon (1959), Eckenfelder (1961), Phillips (1961) and others. A non-Newtonian system, such as exemplified by a suspension of paper pulp, showed a reduction in the SOR by 75-85% (see Tables 24, 25 and 26). The data show that an increase in agitation speed is a more effective technique for improving oxygen transport than by a corresponding increase in aeration rate. This is true regardless of the presence or absence of suspended solids.

Table 24:

Sulfite oxidation rates in the absence of suspended solids and antifoam. Fermentor was operated at constant stirring and variable aeration rates

Agitation rate, rpm	Aeration rate		SOR*	Aeration eff'cy. nominal %
	litres/min	mM O_2/ℓ-min		
300	0	0	0.005	-
300	2	1.5	0.19	13
300	5	3.8	0.31	8
300	8	6.2	0.39	6
300	10	7.7	0.45	6
300	15	11.5	0.51	4

Table 25:

Sulfite oxidation rates in the absence of suspended solids and antifoam. Fermentor was operated at constant aeration and variable stirring rates

Agitation rate, rpm	Aeration rate		SOR*	Aeration eff'cy. nominal %
	litres/min	mM O_2/ℓ-min		
0	5	3.8	0.03	1
300	5	3.8	0.31	8
500	5	3.8	0.97	25
700	5	3.8	1.73	45

*sulfite oxidation rate in mMO_2/ℓ-min.

Table 26:

Sulfite oxidation rates in the presence of 0.9% paper pulp. Fermentor was operated at constant aeration and variable stirring rates

Agitation rate, rpm	Aeration rate		SOR	Aeration eff'cy.
	litres/min	mM O_2/ℓ-min	-	nominal %
300	5	3.8	0.05	1
400	5	3.8	0.09	2
500	5	3.8	0.17	4
600	5	3.8	0.30	8
700	5	3.8	0.41	11

Table 27:

Sulfite oxidation rates in the presence of 0.1% carboxy methyl cellulose* (Carboxel X-385). Fermentor was operated at variable aeration and stirring rates

Agitation rate, rpm	Aeration rate		SOR	Aeration eff'cy.
	litres/min	mM O_2/ℓ-min		nominal %
300	5	3.8	0.26	7
300	10	7.7	0.36	5
500	5	3.8	0.83	22

*Apparent viscosity of 0.1% solution of Carboxel X-385 was 40 cps at 60 rpm with #1 spindle

Table 28:

Sulfite oxidation rates in the presence of cellulose powder (Alpha-floc)

Alpha-floc, % w/v	Agitation rate, rpm	Aeration rate		SOR	Aeration eff'cy. nominal %
		ℓ/min.	mM O_2/ℓ-min		
0.4	300	5	3.8	0.30	8
0.6	300	5	3.8	0.29	8

Table 29:

Sulfite oxidation rates in the presence of 0.9% paper pulp and antifoam (Hodag M-8). Fermentor was operated at variable aeration and stirring rates

Hodag M-8 ppm	Agitation rate, rpm	Aeration rate		SOR	Aeration eff'cy. nominal %
		ℓ/min.	mM O_2/ℓ-min		
83	300	5	3.8	0.09	2
166	300	5	3.8	0.10	3
166	500	5	3.8	0.45	12
166	300	10	7.7	0.11	1

More specifically, when suspended solids were absent, the SOR increased approximately as the square root of the aeration rate (see Table 24), but when paper pulp solids were present, the SOR remained essentially constant in response to a 100% increase in the aeration rate (see Table 29) at constant stirring rate. On the other hand, at constant aeration rates the SOR varied approximately as the square of the agitation rate when suspended solids were absent and, as the cube of the agitation rate when the paper pulp solids were present (see Tables 25 and 26). In other words, when paper pulp solids are suspended in sulfite solution, the oxygen transfer rate from the air bubbles to the liquid phase increases proportionately to power input. Maxon (1959) came to a similar conclusion from his aeration-agitation studies on the non-Newtonian novobiocin fermentation system. Nevertheless, there is a serious lack of agreement amongst investigators using different, and, indeed, the same methods for determining oxygen uptake rates. Roxburgh (1962) even went so far as to suggest that no general relationship exists between the SOR and the transfer coefficient for oxygen from air to the fermentation media.

As a general rule, the sulfite method gives higher volumetric transfer coefficients '$k_L a$' than the popular

gassing-out* method, because of the smaller bubbles formed in the sulfite electrolyte solution. Taguchi and Humphrey (1966) suggested a direct method for determining volumetric transfer coefficients utilizing the actual fermentation system. This would appear to offer the soundest basis for scale-up of aerobic fermentation processes.

Cellulose powder (Alpha-floc) at 0.4 and 0.6% suspension did not seriously reduce the SOR, (see Table 28). Brierley and Steel (1959) in a similarly oriented experiment, showed that a 3% suspension of sago pellets, to simulate mold pellets, did not reduce the oxygen transport significantly. The physical dimensions of sago pellets and cellulose powder are substantially isometric, and suspensions of these solids are easily fluidized. The fluid behaviour pattern of these systems can be characterized as Newtonian.

The presence of antifoam agent marginally improved the SOR of the paper pulp suspension; most probably due to a reduction of the size of the air bubbles (see Tables 26 and 29). Eckenfelder and Barnhart (1961) showed that very small concentrations of surface active agents markedly decreased the oxygen transport coefficient. Higher concentrations of surfactants resulted in improved oxygen transport, presumably due to a decrease in bubble size.

*Gassing-out method: The water or broth in the fermentor is purged with nitrogen gas to a low level of dissolved oxygen. Air or oxygen is then introduced into the liquid and the increase in dissolved oxygen is measured against time. The k_La value is determined from $dc/dt = k_La\ (c^*-c)$.

The addition of 0.1% carboxy methyl cellulose as a soluble polymer to impart non-Newtonian flow characteristics to the aqueous solution in the fermentor, resulted in a reduction of the SOR by approximately 15% for a seemingly modest increase in apparent viscosity to 40 cps (see Tables 24 and 27).

Each of the Tables in this sulfite oxidation study includes values for the nominal aeration efficiency. The aeration efficiency was reported as the percentage of the oxygen in the air supply that was absorbed in the sulfite solution. (Note: the sulfite technique presupposes that all the absorbed oxygen immediately reacts with the sulfite and that for all intents and purposes the dissolved oxygen concentration in the liquid phase is zero). Because air is normally supplied to an aerobic fermentation in large excess, the aeration efficiency is not as useful a statistic as the actual mass transfer rate; however, it is included in the data for comparison with the aeration efficiencies of the glucose - glucose oxidase system which are reported in a later section.

Shu (1953) noted that the peak oxygen demand for an active culture of Aspergillus niger falls in the 0.5-1.0 mM O_2/litre-min range, and because Aspergillus oryzae belongs to the same genus of filamentous fungi, it is not unreasonable to expect this organism to have a peak oxygen demand within a similar range. Solely on the basis of SOR data reported above,

the capability of the 14-litre bench fermentor to meet the peak oxygen demands of the Aspergillus oryzae fermentation is questionable; particularily in view of the fact that oxygen transfer rates as determined by the sulfite method are generally higher than might be measured under actual fermentation conditions.

CHAPTER 6: DEVELOPMENT OF A PRACTICAL HYDROGEN PEROXIDE - CATALASE SYSTEM FOR OXYGENATION OF AN ASPERGILLUS FERMENTATION.

The fermentation studies that are detailed in this chapter develop the basic experimental program leading to a practical technique for the oxygenation of a non-Newtonian fermentation with Aspergillus oryzae. Superimposed on the biological problems inherent in such an endeavour were the additional electronic and mechanical problems associated with agitation and temperature control of the fermentor.

Perhaps the most vexatious misadventure was the frequent failure of the shear pin connecting a flexible cable from the motor to the agitation shaft on the bench fermentor. It became increasingly obvious that the selection and arrangement of the components in the fermentor control assembly needed much improvement. For example, the flexible cable connecting the motor to the agitation shaft was bent through an angle of 180° in spite of the recommendation for a maximum bend of 90° by the cable manufacturer. The transmission of power through 180° caused a slight but, nevertheless, perceptible whip at each revolution. Regardless of the many types and sizes of shear pins used, each eventually failed under the continual stress. Needless to say, the usefulness of data from some of the stirred fermentations was entirely negated or at least

diminished by the failure of the agitation mechanism. It was not until the motor was mounted horizontally on top of the fermentor control console and the power transmitted to the agitator shaft through a 90° gear box, that reliable agitation performance was established.

6.1 Establishment of the General Behaviour Pattern of Stirred Fermentations

Initially, several stirred fermentations were undertaken to obtain some information on the gross cultural characteristics of Aspergillus oryzae in the standard fermentation medium. Problems to be evaluated were: severity of the foam problem, mycelial growth in pellet or filamentous form, mycelial distribution in the media and the relationship of protease production to growth.

As reported in Section 4.5, the use of tap water for media make-up was not found favourable and was replaced by distilled and/or de-ionized water. This was confirmed in both shake flask and stirred fermentation studies. Discrete pellet formation was never observed in any of the stirred fermentations regardless of the presence or absence of Carbopol in the medium. Consequently, the addition of Carbopol to fermentation media used in the stirred fermentor was discontinued.

One of the difficulties encountered in the stirred fermentations was in the collection of a representative sample of the fermentor contents for biomass determination. Mycelia tended to adhere to solid surfaces such as thermometer wells, sample and sparger tubes, agitator shaft and baffles, and, furthermore, the mycelia formed a fixed mat around the glass wall of the fermentor jar at the surface of the fermentation. For these reasons, the mycelial solids concentrations - as determined from free flowing samples syphoned from the fermentor - were marginally lower than the actual values; this was particularly true during the period of maximum biomass content.

The pH profile of the stirred fermentation cycle was similar to that observed from the shake flask studies. There was an initial drop of about 1 pH unit during the first day or so of the fermentation cycle followed by a steady increase over the balance of the cycle to a maximum pH in the 6.3-6.7 range.

A standard foam pattern was observed in the stirred fermentations undergoing active aeration. An aggressive foam head developed in the fermentation during the time interval between the first and second day and gradually subsided with time in the latter half of the cycle. The reasons for this are not clear, but are probably related (1) to poor air-bubble dispersion as the level of suspended solids increased and (2) to the presumed destruction and/or metabolism of foam

inducing substances. The addition of 500 ppm Hodag M-8 antifoam agent to the fermentation medium prior to sterilization, effectively controlled the foam during the fermentation cycle regardless of the aeration rate that was selected.

6.2 The Effect of Oxygen in the Stirred Fermentation

If it could be shown that the yield of product was a function of the oxygen supply to the culture during a critical period of the fermentation, as, indeed, Virgilio (1964) so aptly demonstrated in his rifamycin fermentations, then the attempted development and application of the enzymatic HPC system to release oxygen in situ would be justified. The role of oxygen on final yield and length of cycle was examined in three separate fermentation studies.

6.2.1 First study examining the role of oxygen in the stirred fermentation

A fermentation was effected in the stirred fermentor under the following operating conditions:

volume of medium	14 litres
agitation speed	315 rpm
aeration rate	2 litres/min
temperature	32°C.

The maximum protease titre was 291 mU/ml in 164 hours. Two samples of the stirred fermentor, taken at 28 hours and placed on the shake table, produced protease titres of 390 and 480 mU/ml respectively in 136 hours; though it was obvious from the 'soupy' condition of the mold that the optimum time for the peak assay was somewhat earlier. In this experiment the improved enzyme titres and shortened cycle of the more vigorously aerated shake flask fermentations indicated that the product yield was predicated on an adequate supply of oxygen to the culture.

6.2.2 The effect of pure oxygen in the stirred fermentation

Two stirred fermentations were undertaken; one control and one test, in which the impact of pure oxygen on overall fermentation performance was examined in a superficial way. The operating conditions imposed on each fermentation were as follows:

volume of medium	11 litres
agitation speed	400-600 rpm
temperature	32°C
aeration of control	2-4 litres air/min
oxygenation of test	air as above replaced by pure oxygen at 0.8-1.6 litres/min for 36 hours during the period of peak mycelial development.

The peak protease titres and the respective fermentation times are indicated in Table 30 below.

Table 30:

The effect of pure oxygen on fermentation time and protease titre

	Fermentation time, hours	Protease titre, mU/ml
control, air only	109	287
test, pure O_2 for 36 h	70	373

As indicated in the previous experiment, an improved protease titre and a shortened fermentation cycle resulted from an increased supply of oxygen to the culture during the growth phase.

6.2.3 A third study on the fermentation response to oxygen

This fermentation experiment was somewhat similar to, but more elaborate than, the previous one in which pure oxygen was substituted for air during part of the fermentation cycle. The modified nitrate-free medium, described later in this chapter, was used. The actual sparge rate of the pure oxygen was predicated on the dissolved oxygen indication of the electrode inserted in the fermentor contents. There was no separate stirred fermentation control in this experimental plan, however, the effect of pure oxygen on the final yield was observed by removing samples from the fermentor immediately

before and after the introduction of pure oxygen. These samples were placed on the shake table for the balance of the fermentation cycle. The conditions of the fermentation were as follows:

volume of medium	14 litres
agitation speed	355 rpm
temperature	32°C
air/oxygen regimen	0-36 h air at 2 litre/min
	36-64 h pure O_2
	64-75 h air at 1 litre/min
	75-114 h anaerobic.

During the introduction of pure oxygen, the dissolved oxygen level was not maintained at a constant value but was permitted to fluctuate widely between 1 and 9 ppm depending on the oxygen demand rate of the culture and the oxygen supply rate; which in point of fact was frequently changed to elicit a response on the dissolved oxygen analyser. The pure oxygen supply rate ranged from a high of 1.3 litres/min during the first half of the sparge period, to a low of 0.22 litres/min during the latter half. After the supply of pure oxygen was discontinued, air was re-introduced at half the former rate for eleven more hours and then turned off. Anaerobic conditions, except for traces of oxygen from the head space gases, prevailed for the balance of the fermentation. Protease titres of the stirred fermentor contents and those of the two shake flask samples, taken before and after the pure oxygen sparge, are shown below in Table 31.

Table 31:

Protease titres and fermentation times of shake flask samples taken from a stirred fermentor before and after the introduction of pure oxygen

	Protease titre, mU/ml			
	84 h	96 h	114 h	156 h
Shake flask: pre-oxygen	12		290	456
Shake flask: post-oxygen	425		650	580
Stirred fermentor	365	372	375	

The volumetric oxygen demand rate was determined several times during the fermentation. The procedure for determining the oxygen demand rate was as follows: the supply of oxygen or air to the fermentation was adjusted to ensure a dissolved oxygen concentration well above 1 ppm, then the source of oxygen was turned off and the decrease in dissolved oxygen concentration between two appropriate values was timed. The volumetric oxygen demand rate was observed to peak at 0.19 mM O_2 litre-min at about 50 hours, however, the specific oxygen demand showed a steady decrease from 0.035 to 0.01 mM O_2/gram-min during the 38 to 75 hour interval of the fermentation cycle. The oxygen demand rates, biomass concentrations and protease titres of the stirred fermentor, in addition to the protease titres of the shake flask samples, are plotted against fermentation time in Figure 7.

The results of the above study demonstrate the impact on yield and fermentation time of hyper-critical oxygen tension during the peak demand period of the fermentation cycle. The indication that the protease titre in the stirred fermentor sample failed to increase appreciably after anaerobic conditions were invoked at 75 hours, suggests the need for a sustained supply of oxygen during the end-fermentation period. It is, indeed, probable that the adverse effect of anaerobic conditions reflected the accumulation of carbon dioxide in the fermentation broth and that some form of aeration or oxygenation was required to ventilate the fermentor contents.

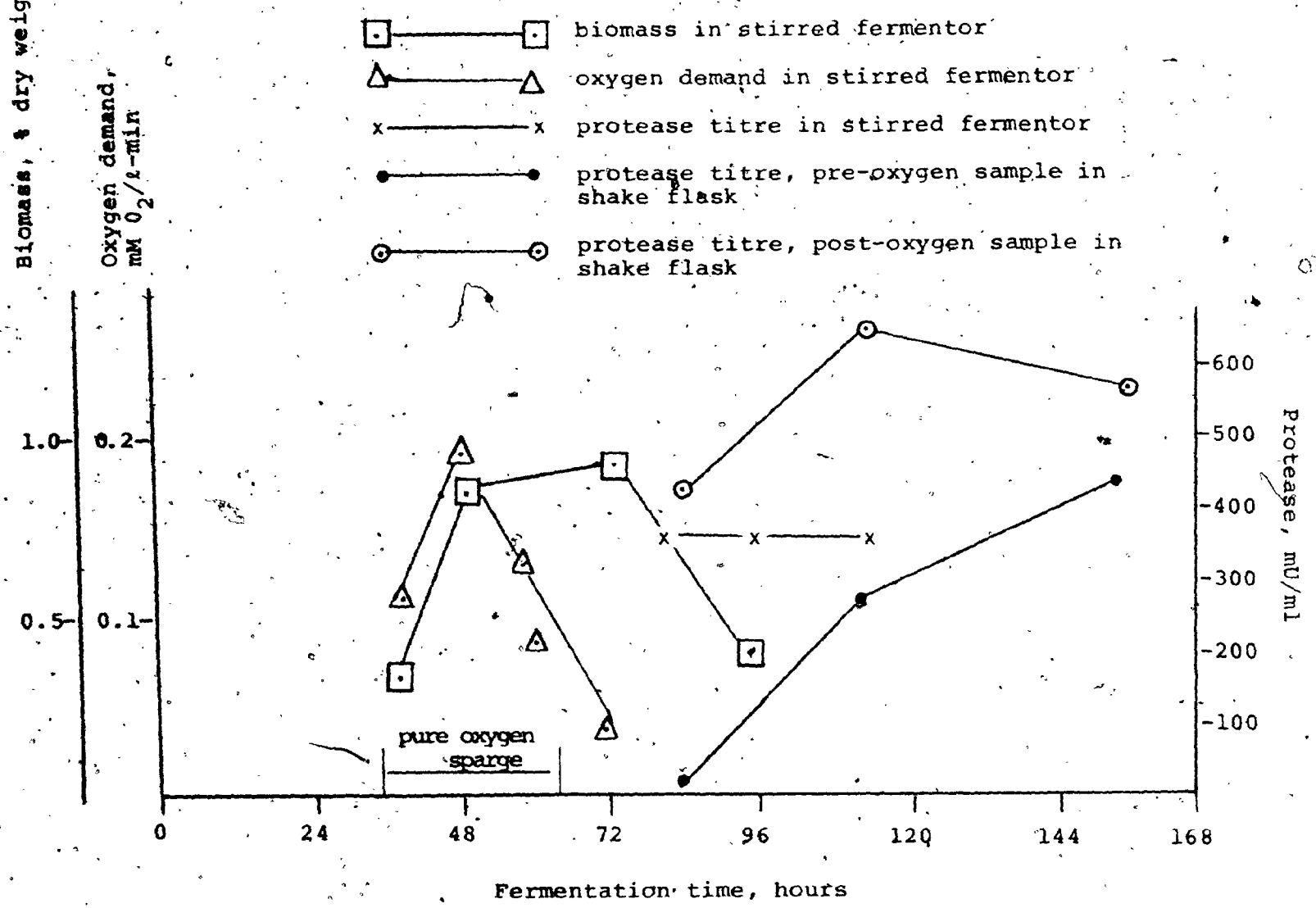


Figure 7. Effect of pure oxygen sparge for 28 hour interval on yield and fermentation time on the culture of Aspergillus oryzae.

6.3 The Response of the Oxygen Analyser to the Release of Oxygen by the HPC Technique

Before undertaking any fermentation experiments involving the enzymatic release of oxygen, it was necessary to investigate the response of the oxygen analyser to discrete additions of catalase and hydrogen peroxide to an aqueous medium. Two essentially similar tests were carried out in the stirred fermentor.

6.3.1 First experimental procedure

The operating conditions in the stirred fermentor were as follows:

volume	12 litres
aqueous medium	tap water, weakly buffered with sodium phosphate + trace minerals of the standard fermentation medium
pH	7.4
agitation	400 rpm
temperature	30°C.

The oxygen analyser was standardized with air and the electrode tip was positioned opposite the upper turbine on the agitator shaft. Sodium sulfite (1 gram) was added to reduce the dissolved oxygen concentration in the water to a low value which was well below the saturation limit of air. (Note: the equilibrium

solubility of oxygen at 157 mm Hg in water at 30°C is 7.5 ppm). When the dissolved oxygen level reached 2.6 ppm, commercial catalase* solution and 30% H_2O_2 were added to give 83 ppm and 50 ppm respectively, in the stirred solution. This was sufficient H_2O_2 to release approximately 24 ppm oxygen into the water.

The oxygen analyser recorded an increase in the dissolved oxygen level at a rate equivalent to 0.06 ppm/min. Additional doses of catalase and peroxide increased the rate of accumulation of the dissolved oxygen. The total H_2O_2 added was sufficient to supply 72 ppm oxygen to the water, however, the maximum dissolved oxygen concentration failed to exceed 21 ppm because of losses from the supersaturated solution to the atmosphere. Figure 8 shows the accumulation of dissolved oxygen in a time-release plot transcribed from the strip chart recorder.

*Beef liver catalase, technical, from Nutritional Biochemicals Corp. This was some old laboratory stock of uncharacterized activity.

6.3.2 Second experimental procedure

The following modifications of the operating conditions were introduced into this test procedure.

volume	11 litres
aqueous medium	unbuffered tap water
pH	7.6
agitation	300 rpm
temperature	22°C.

The oxygen electrode was standardized and positioned as outlined in the previous test. Sodium sulfite (1 gram) was added to reduce the dissolved oxygen level. When the dissolved oxygen level reached a concentration of 2.5 ppm, freshly acquired catalase* solution and H_2O_2 solution were added to give 45 ppm and 43 ppm, respectively. The release of oxygen was more rapid on this occasion than on the first because of the more active catalase solution. Sufficient H_2O_2 was added initially to supply 20 ppm oxygen to the water and the oxygen analyser recorded 82% of this increase during the first hour. An additional amount of H_2O_2, equivalent to 80 ppm dissolved oxygen, was added to the stirred fermentor at the end of the first hour and the dissolved oxygen

*Beef liver catalase solution, technical, from Nutritional Biochemicals Corp., Cleveland, Ohio.

concentration increased to approximately 28 ppm during the next 1.5 hours and then started to decline. A plot of dissolved oxygen concentration versus time obtained with a dissolved oxygen probe and recorded on a strip chart, is presented in Figure 9.

The results of the above tests clearly established the ability of the oxygen analyser to measure and record the enzymatic release of oxygen due to the interaction of H_2O_2 and catalase in tap water. It was evident from the recorder tracings that the rate of accumulation of dissolved oxygen is a function of the substrate concentration, enzyme activity and degree of oxygen saturation of the water, particularly when temperature and agitation are held constant.

Conditions of Test	
operating volume	12 litres water
agitation rate	400 rpm
temperature	32°C
pH	7.4

a ..... 1 g of Na_2SO_3 added
b ..... 83 ppm catalase solution and 50 ppm H_2O_2 added
c ..... 83 ppm catalase solution added
d ..... 50 ppm H_2O_2 added
e ..... 50 ppm H_2O_2 added

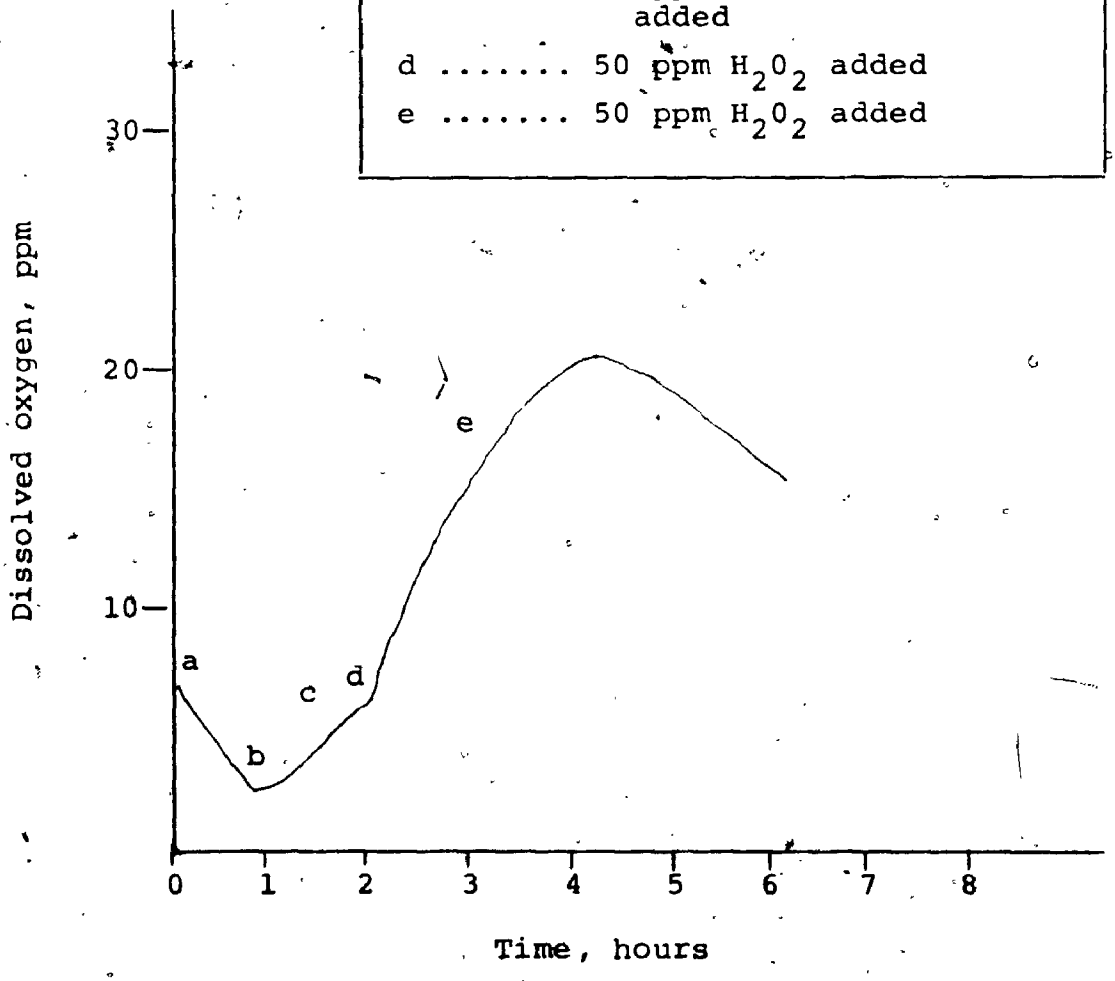


Figure 8. The release of dissolved oxygen in response to discrete additions of catalase solution and hydrogen peroxide to water in the stirred fermentor

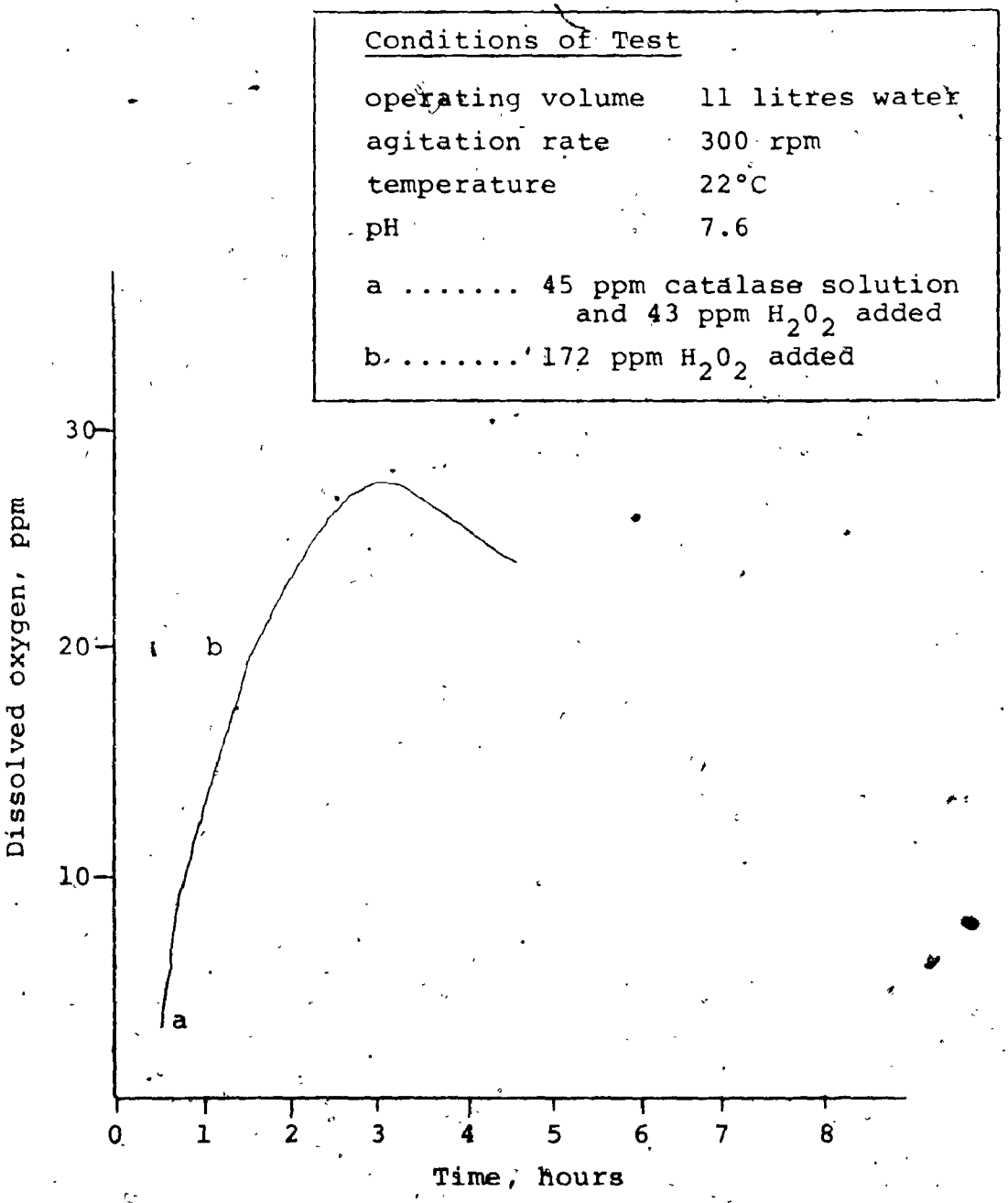


Figure 9. The release of dissolved oxygen in response to discrete additions to the stirred fermentor of freshly acquired catalase solution and hydrogen peroxide

6.4 The Stability of Hydrogen Peroxide in the Standard Fermentation Medium

If H_2O_2 had a marked affinity for any of the ingredients in the culture medium, the effective use of the HPC oxygenation technique would be negated. Samples (1 litre) of the standard fermentation medium were adjusted with NH_4OH to three different pH values within the normal fermentation profile. Approximately 1900 ppm H_2O_2 was added to each sample prior to incubation at 31°C for 36 hours. Aliquots of the peroxide dosed fermentation medium were analysed for H_2O_2 content at appropriate intervals. The values are recorded in Table 32 below.

Table 32:

The time-decay of hydrogen peroxide in the standard fermentation medium

	H_2O_2, ppm (pH)				
Flask #	0 h	2 h	4 h	12 h	36 h
1	1895 (4.6)	1865 (4.6)	1800 (4.6)	1520 (4.6)	605 (4.2)
2	1880 (5.4)	1800 (5.4)	1740 (5.4)	1450 (5.3)	725 (4.6)
3	1865 (6.1)	1770 (6.1)	1635 (6.0)	1070 (5.9)	440 (5.5)

Considering the relatively high concentration of H_2O_2 in the medium and the propensity of this chemical to act as an oxidizing agent, the short term stability was surprisingly good; especially at the lower pH registers. Approximately 98% of the H_2O_2 activity was recovered after two hours at pH

4.6. This would suggest an element of safety for a practical HPC system operating at very low steady state peroxide concentrations. Prolonged contact of H_2O_2 with the medium tended to lower the pH; most probably reflecting an oxidation effect. Fortunately, the pH of the fermentation during the expected oxygenation period falls within a favorable stability range.

6.5 The Tolerance of Aspergillus oryzae to Hydrogen Peroxide

Some indication of the tolerance of Aspergillus oryzae to the presence of H_2O_2 was required in order to determine permissible steady state concentrations of this germicidal agent in the culture medium. A preliminary set of shake flask fermentations containing 0, 150, 300, 600, and 1200 ppm H_2O_2 in the standard fermentation medium, produced normal mycelial growth in all flasks, however, the final protease titres at 111 hours were zero. The fact that mycelia rather than spores were used to inoculate unsterile fermentation medium, undoubtedly accounted for the lack of protease activity.

The above results were sufficiently encouraging to warrant a more critical examination of the toxicity of H_2O_2 to Aspergillus oryzae. H_2O_2 was added to sterilized fermentation medium over a concentration range of 0-1000 ppm. Carbopol 940 at 200 ppm was added to half the shake flasks. A standard spore suspension was used to inoculate the flasks which were subsequently fermented in shake flasks at 31°C for 120 hours. The results of these fermentations are recorded in Table 33.

Table 33:

The toxicity response of Aspergillus oryzae to various concentrations of hydrogen peroxide in shake flask fermentations

Flask #	H_2O_2, ppm	Carbopol	Final pH	Protease titre, mU/ml
1	0	-	6.50	516
2	0	-	6.45	399
3	0	-	6.40	418
4	0	-	6.35	381
5	0	-	6.40	484
6	0	+	6.65	348
7	0	+	6.50	565
8	0	+	6.65	459
9	0	+	6.70	546
10	0	+	6.65	378
11	200	-	6.60	336
12	200	+	6.65	443
13	500	-	6.50	372
14	500	+	6.55	381
15	1000	-	4.35	0
16	1000	+	5.80	86

Surprisingly, the spores were able to germinate in the standard fermentation medium containing 1000 ppm H_2O_2. At 200 ppm, spores germinated at the same time as the controls, though the spores in the flasks with 1000 ppm peroxide germinated two days later. Protease titres in the flasks with 200 and 500 ppm H_2O_2 compared favorably to the controls, and the poor titre in the flasks with 1000 ppm H_2O_2 might reflect, in part, the late

start in germination. A subsequent fermentation at the identical peroxide concentration of 1000 ppm produced a maximum titre somewhat less than half the control.

The results of this experiment were quite encouraging and gave considerable latitude in the steady state peroxide concentration that could be tolerated in the application of a HPC oxygenation technique.

6.6 Initial Assessment of the HPC Technique as a Source of Oxygen for a Fermentation

At this early state in the development of the HPC oxygenation technique, an in vitro reactor experiment was undertaken to test the gross response of the stirred fermentor to the novel oxygenation system.

It was assumed that the 8.8% H_2O_2 solution to be used for infusion to the fermentor was sterile and no special precautions were undertaken to sterilize this solution. The catalase solution (0.5% dilution of original stock material) was sterilized by passage through a Millipore filter fitted with a cellulose nitrate membrane. The catalase and H_2O_2 solutions were infused into the fermentor beginning at the 30.5 hour of the fermentation cycle. This experiment was discontinued when test samples indicated an accumulation of H_2O_2 in the fermentor.

Though at the time of this experiment it was not known that nitrate ion in the fermentation medium markedly reduced the activity of catalase, the most probable cause for the failure of the experiment was attributed to the adsorption of significant amounts of catalase on the surface of the Millipore membrane. This possibility was examined by measuring the volume of oxygen released in the catalase assay apparatus by samples of unfiltered and Millipore-filtered catalase solutions. The unfiltered catalase solution was observed to possess six times the activity of the filtered solution and supported the contention that a significant proportion of the enzyme activity was removed during the filtration operation. The search for a satisfactory membrane of different chemical and adsorption properties was not pursued.

6.7 The Development of a Satisfactory Technique for Sterilizing the Catalase Infusion Solution

It was very important that a relatively simple and practical technique be developed for the preparation of sterile catalase solutions. The application of heat was categorically out of the question, and in the interest of a speedy resolution of the problem, filtration was temporarily rejected in favor of chemical sterilization by formaldehyde. The use of formaldehyde was considered promising because of its well-known germicidal and fungicidal properties and

because it is under certain conditions an extremely mild protein denaturant. For example, formaldehyde converts diptheria toxin to toxoid without interfering with the antigenic response of the protein.

The problem at hand was not whether formaldehyde could chemically sterilize the catalase solution, but whether in so doing it destroyed or inhibited the catalase activity. An initial check with a 0.5% dilution of the stock catalase solution, to which 370 ppm formaldehyde were added, showed no immediate loss of activity when compared to a control. This was encouraging, however, there was some concern that the pH of the catalase solution might have some effect on its stability and/or its reactivity. Tests showed that under identical concentration of reactants, the ability of catalase to release oxygen from H_2O_2 at pH 3.8 was approximately half that at pH 6.9. Other tests demonstrated that there was little, if any, differences in the reaction rate within the pH 4.4-6.8 range. Information suggested that it would be prudent to buffer the formalized-catalase solution at pH 6.5 or thereabouts. Since tests showed that phosphate was compatible with catalase, the following carrier solution was formulated:

distilled water	1 litre
KH_2PO_4	3 g
K_2HPO_4	2 g
formalin (37% H_2CO)	2 ml
pH	6.5.

Catalase added to the above carrier solution was found to be sterile and stable, at least for a period of time likely to be encountered in most industrial fermentation cycles. In one particular evaluation, no loss of catalase activity was detectable after five days storage at room temperature. In two other evaluations there was some indication of enhanced catalase activity with the passage of time. The results of these latter tests are shown in Table 34. The stock carrier solution contained 250 ppm of crude beef liver catalase powder (Nutritional Biochemical Corp.). The activity checks were carried out at ambient temperature in a reaction flask containing the equivalent of 800 mg H_2O_2 and 0.25 mg catalase (as powder) in a total reaction volume of 100 ml.

The apparent increase in catalase activity was not examined in any more detail. It was clear, however, that a simple and practical technique was now available for the preparation of a sterile and stable catalase infusion solution.

Table 34:

Activity checks of formalized-catalase solution after storage at room temperature for discrete time periods

Sample #	Sample age, days	Time for release of 100 ml O_2, minutes	Volume O_2 released in 20 min, ml
1	0	6	140
1	5	5.25	169
2	0	6.75	122
2	11	5.5	141
2	171*	12	107

*Some mold was observed to be growing in the formalized-catalase solution.

6.8 The Tolerance of Aspergillus oryzae to Formaldehyde

Since formaldehyde had been selected as a satisfactory chemical sterilant for the catalase infusion solution, the next problem involved a determination of the tolerance of the Aspergillus oryzae culture to the presence of formaldehyde in the fermentation medium. To complete this study, a series of shake flask fermentations was undertaken in which several concentrations of formaldehyde with and without H_2O_2 were added to the standard fermentation medium prior to the inoculation with spores. The temperature of the fermentations was controlled at 31°C. The specific treatments and the respective protease assays after 134 hours into the fermentation cycle are given in Table 35 below.

Table 35:

The fermentation response of Aspergillus oryzae to various concentrations of formaldehyde, with and without the addition of hydrogen peroxide.

Flask #	Treatment		Protease titre, mU/ml	Remarks
	HCHO ppm	H_2O_2 ppm		
1	0	0	398	
2	0	0	415	
3	74	0	533	
4	148	0	25	pellets, pH 4.6
5	370	0	0	no growth
6	74	200	525	
7	148	400	332	
8	370	1000	0	no growth
9	740	200	0	no growth

Compared to the controls, flasks #3 and #6 required an additional twelve hours to germinate, whereas flasks #4 and #7 required an additional 48 hours. The results indicated that Aspergillus oryzae spores could germinate and grow well in fermentation medium containing 74 ppm formaldehyde and, indeed, the final protease titres surpassed those of the control. Formaldehyde at twice the above concentration permitted normal growth and yielded a modest protease titre in spite of the inordinately long germination period.

To ensure that the formaldehyde concentration in the fermentor does not exceed 74 ppm, some adjustments in the catalase concentration and/or flow rate of the infusion solution might be necessary at times. However, this does not appear to be a serious constraint. Indeed, low levels of formaldehyde in the fermentation medium could offer significant protection against adventitious bacterial contamination.

6.9 Second Assessment of the HPC Technique as a Source of Oxygen for a Fermentation

Having demonstrated that the culture of Aspergillus oryzae could tolerate the presence of 200 ppm H_2O_2 and 74 ppm of formaldehyde without compromising the final yield of protease, a repeat of the experiment outlined in Section 6.6 was planned to test the response of the fermentation to the modified HPC oxygenation system.

Ten litres of standard fermentation medium were seeded with Aspergillus oryzae spores in the stirred fermentor which was maintained at a constant temperature of 30°C. Agitation and air-sparge rates were increased from relatively low values to higher values of 300 rpm and 5 litres/min respectively, during the first twelve hours. At 15.5 hours, when there was visible evidence of mycelial growth, the air was turned off and a 9% H_2O_2 solution and a 1% dilution of chemically sterilized (740 ppm formaldehyde) beef liver catalase solution were infused into the fermentor at identical rates through separate channels. The system was operated to supply 0.79 mM O_2/litre-min initially for 25 minutes, then at 0.16 mM O_2/litre-min for the next 4.25 hours.

Since there was little, if any, evidence of new mycelial elaboration during the infusion period, the fermentor contents were sampled and checked for H_2O_2 content. The concentration of H_2O_2 in the fermentation medium was found to be 1540 ppm indicating a marked inhibition in catalase activity. The decay of H_2O_2 in the fermentor was followed during the next sixteen hours (Figure 10). It was evident that whatever residual catalase activity that was present in the fermentor at the termination of the infusion period, degenerated over the next hour or so. The activity of the catalase infusion solution was checked by the standard assay technique and was found to be more than adequate to handle the peroxide loading. The rate of release of oxygen at two different concentrations of

hydrogen peroxide did not support the possibility that toxic levels might accumulate in the fermentor. The inordinately high concentration of peroxide in the fermentor could only be explained in terms of an agent or agents in the fermentation medium that were toxic to the catalase.

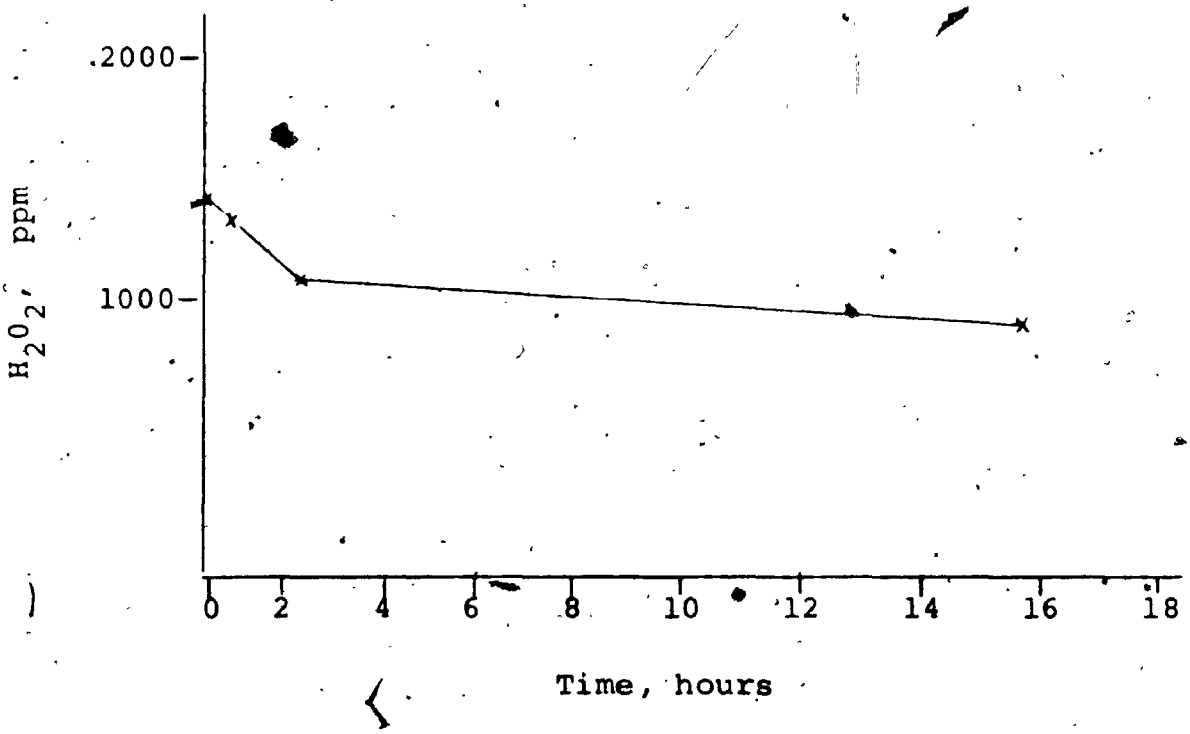


Figure 10. The decay of H_2O_2 in the fermentor after the flow of HPC solutions to the fermentation medium was interrupted. H_2O_2 had accumulated due to inhibition of the catalase

6.10 The Identification of the Catalase Inhibitor

The research project had reached an impasse and could not continue until the catalase inhibitor in the fermentation medium was identified and eliminated.

A preliminary test of catalase activity, in which 90 ml of buffered water was replaced with standard fermentation medium, indicated a release of oxygen at approximately one half the rate of the control. This confirmed the presence of an inhibitory substance in the fermentation medium. The next step involved a series of tests in which specific components of the medium were examined for catalase inhibition using the timed release of oxygen in the assay apparatus as an index of activity. For each test, the active concentrations in the reaction flask of the H_2O_2 and the stock catalase solution were 900 and 200 ppm, respectively. The pH of the reaction mixture was 5.35. Table 36 shows the volume of oxygen released at ambient temperature during a two minute time interval for various combinations of ingredients that are present in the standard fermentation medium.

Table 36:

The timed release of oxygen in the catalase apparatus for various combinations of ingredients that are present in the standard fermentation medium

Flask #	Treatment	O_2 released in 2 min, ml
1	phosphate buffer control	82
2	complete standard medium	36
3	standard medium less minerals and lactic acid	37
4	standard medium less lactic acid	39
5	phosphate buffer + 0.2% KNO_3	35
6	phosphate buffer + 0.1% KNO_3	40
7	standard medium less KNO_3	80

The results clearly implicated the nitrate ion as the inhibitory agent in the standard fermentation medium. A separate test using the antifoam agent, Hodag M-8, at the 3% level showed no evidence of toxicity towards the activity of the catalase.

6.11 Determination of the Concentration Effect of Nitrate on Catalase Activity

This experiment examined the inhibitory effect of KNO_3 on catalase activity at various concentrations. Specific amounts of KNO_3 were added to nitrate-free fermentation medium and each concentration was checked for inhibitory effect on the

catalase in the assay apparatus. The catalase and peroxide concentrations in the reaction flask were similar to those used in the experiment outlined in Section 6.10. The reaction was carried out at pH 5.2 and ambient temperature. The results are given in Table 37 below.

Table 37:

The inhibitory effect of KNO_3 on catalase activity

KNO_3 in medium ppm	Volume of O_2 released, ml	
	2 min	4 min
0	83	124
12	63	97
25	53	82
50	46	70
100	41	58
200	37	50
500	33	41
1000	29	37
2000	24	31

A log-log plot (Figure 11) of the KNO_3 concentrations versus the rate of oxygen released gave an approximate straight line. It was evident that KNO_3 was quite toxic and a concentration as low as 12 ppm had a definite inhibitory effect on catalase activity. Since the standard fermentation medium contained 2000 ppm KNO_3, it was necessary to find an alternative source of nitrogen.

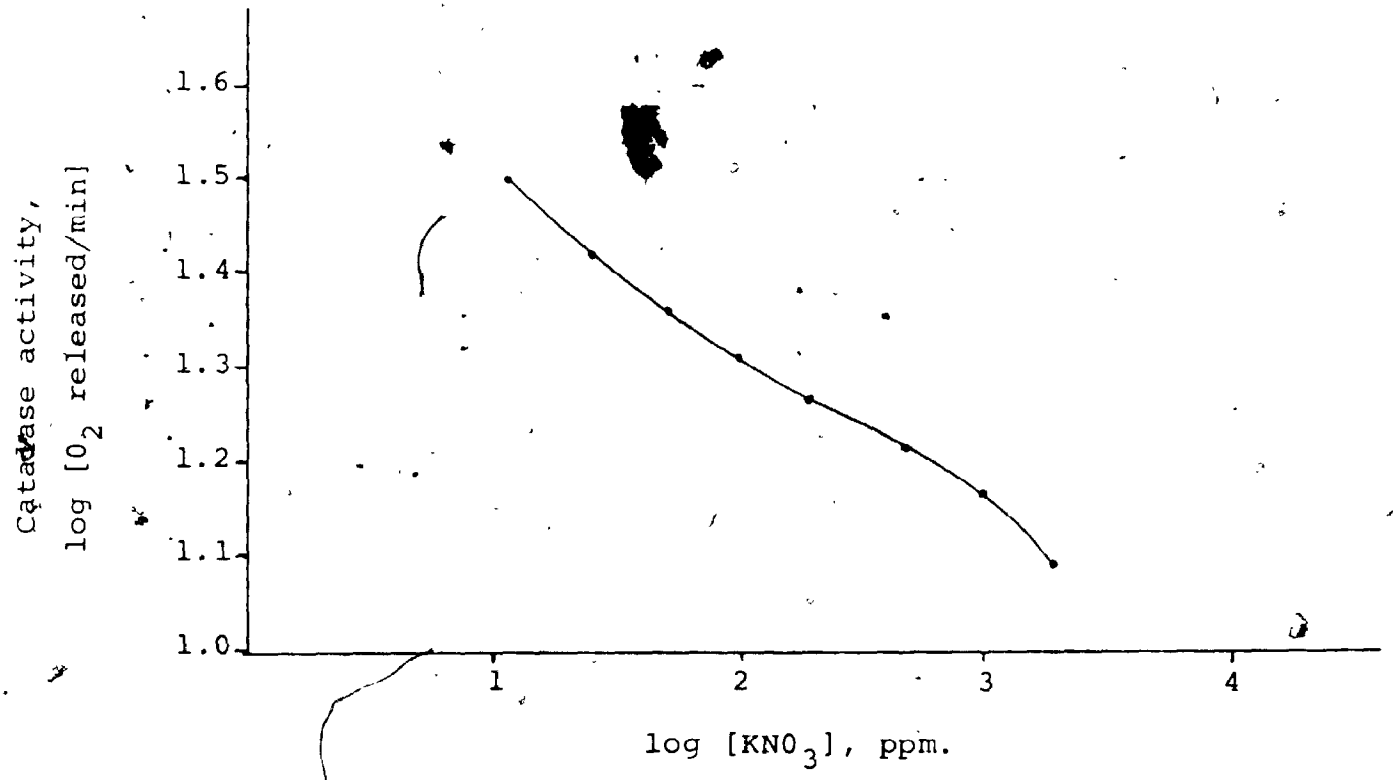


Figure 11. The log-log plot of the nitrate concentration in the fermentation medium versus catalase activity

6.12 The Role of KNO_3 as an Ingredient in the Fermentation Medium

Before the search for a substitute for nitrate was initiated, it was decided to investigate the impact of KNO_3 as an ingredient in the standard medium under fermentation conditions. A series of shake flask fermentations was conducted with media containing a wide range of nitrate concentrations. The pH and protease titres after 104 hours on the shake table at 32°C are shown in Table 38 below.

Table 38:

The response of Aspergillus oryzae to different concentrations of KNO_3 in the fermentation medium

Flask #	KNO_3 ppm	Final pH		Protease titre, mU/ml	
		individual	average	individual	average
1	0	5.90	5.92	139	156
2	0	5.90		180	
3	0	5.95		168	
4	0	5.95		136	
5	50	5.95	5.98	197	172
6	50	6.00		147	
7	100	6.10	6.05	267	256
8	100	6.00		246	
9	200	6.00	6.08	238	248
10	200	6.15		258	
11	500	6.15	6.18	340	346
12	500	6.20		353	
13	1000	6.40	6.40	513	455
14	1000	6.40		398	
15	2000	6.60	6.60	452	488
16	2000	6.60		525	

The average biomass in flasks 1-8 was 0.77% and in flasks 9-16 was 0.71%, indicating that the presence of nitrate in the fermentation medium was not necessary for mycelial development. The final protease titre, on the other hand, appeared to be a function of the nitrate concentration in the medium. The log-log plot of the average protease titres versus the KNO_3 concentrations in each set of fermentations (Figure 13) was observed to be linear. Furthermore, the observation that the final pH of the fermentation appeared to be a direct function of the original KNO_3 concentration in the medium (Figure 12) strongly suggested that the role of KNO_3 was one of pH development to more neutral levels during the end-fermentation phase.

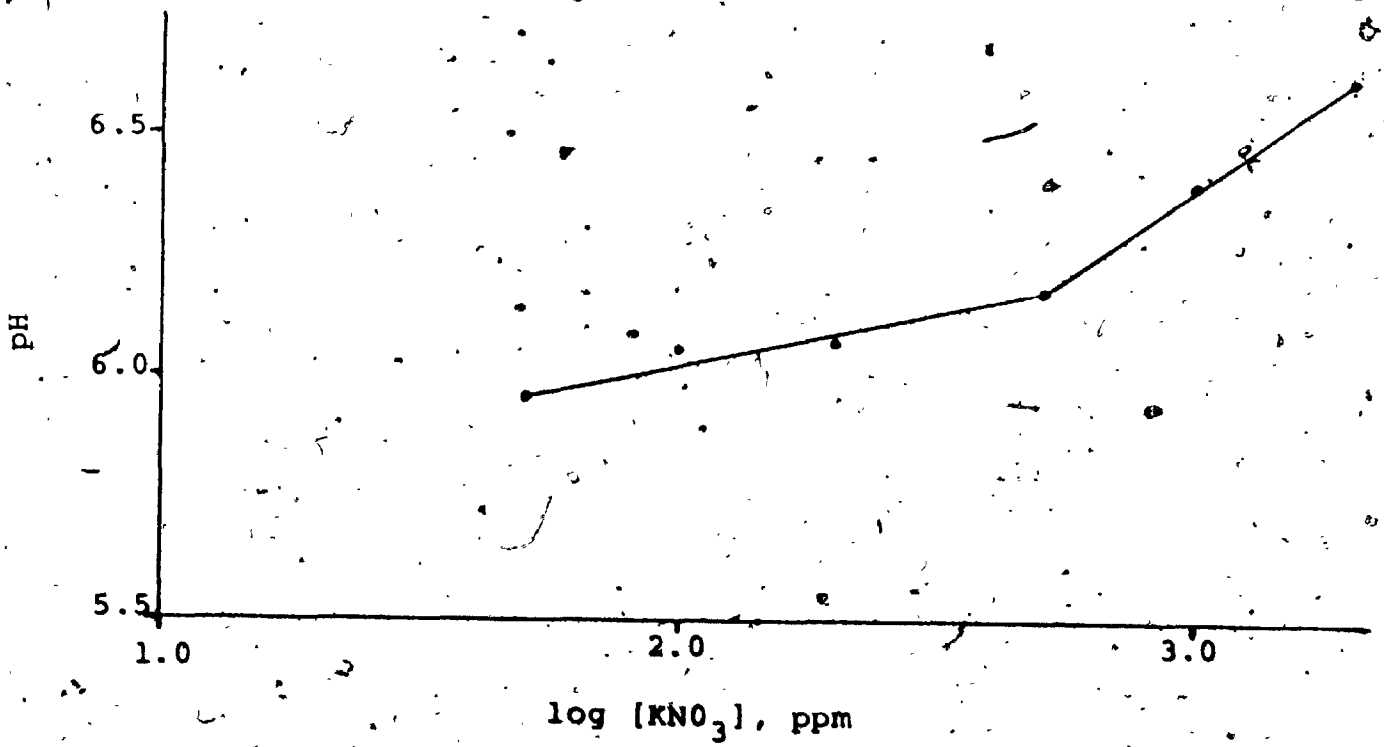


Figure 12. A plot of end fermentation pH versus log of KNO_3 concentration in the medium

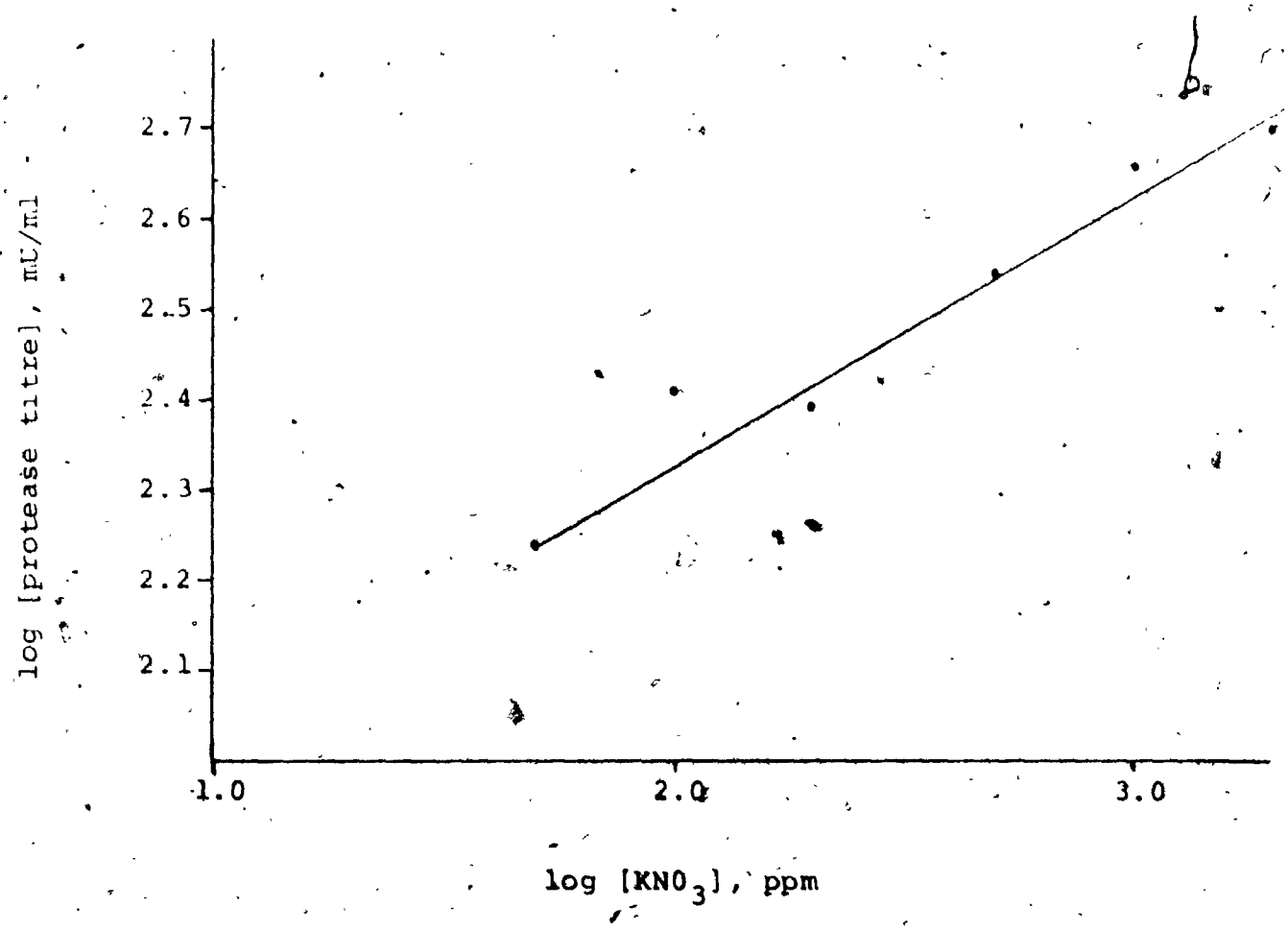


Figure 13. A log-log plot of KNO_3 concentration in the fermentation media versus the protease response of Aspergillus oryzae in shake flask culture

6.13 The Fermentation Response of *Aspergillus oryzae* to the Addition of KNO_3 after the Mycelial Solids have been Developed

The inhibitory effect on the catalase activity could be avoided by delaying the addition of KNO_3 to the fermentation medium until such time as the HPC solutions had been infused into the fermentor. The presence of nitrate during the latter part of the fermentation might mediate a pH environment favorable for protease production. The following experiment was undertaken to answer this question.

A series of shake flask fermentations was conducted on the incubator shake table at 32°C in which the normal level of KNO_3 (2000 ppm) was added at different times during the fermentation cycle. One set received KNO_3 at the beginning of the fermentation cycle, two other sets received KNO_3 at 45 and 61 hours, respectively, into the fermentation cycle and a remaining set did not receive any KNO_3 at all. The flasks were sampled for protease activity at 106 hours and results are indicated in Table 39 below.

Table 39:

Effect of the delayed addition of KNO_3 to the fermentation medium on protease synthesis

Time of KNO_3 addition	Flask #	Final pH		Biomass %	Protease, mU/ml	
		individual	average	average	individual	average
initially	1	6.85	6.79	0.69	533	482
	2	6.80			467	
	3	6.75			471	
	4	6.75			455	
45 h	5	6.50	6.49	0.64	398	395
	6	6.50			385	
	7	6.45			394	
	8	6.50			402	
61 h	9	6.30	6.29	0.68	197	194
	10	6.35			266	
	11	6.25			144	
	12	6.25			168	
none added	13	6.15	6.10	0.76	209	178
	14	6.10			193	
	15	6.00			140	
	16	6.15			172	

The highest protease titres and highest pH values were associated with the earliest KNO_3 additions; a result that was compatible with the data from the previous experiment. Here again, the presence or absence of KNO_3 did not compromise the development of mycelial solids. One could infer from the

data that the primary function of the KNO_3, with respect to protease yield, was one of pH control to the higher registers. The correlation coefficient between the final pH of the fermentation and the corresponding protease titre was 0.99 (see Appendix 2). This statistic supported the evaluation of a non-toxic buffer system as a replacement for nitrate in the fermentation medium.

6.14 Third Assessment of the HPC Technique as a Source of Oxygen for a Fermentation

Since shake flask fermentations demonstrated that nitrate-free media produced a normal quantity of mycelia and a positive, though markedly reduced protease titre, it was considered useful to test the HPC oxygenation system in a nitrate-free fermentation medium and, consequently, the following experimental stirred fermentation was carried out. The operational conditions of the fermentor were as follows:

volume of nitrate-free medium	10 litres
agitation speed	265 rpm
temperature	32°C
air/HPC regimen	0-20 h air at 1-5 ℓ/min
	20-29.5 h air at 5 ℓ/min
	20.5-38.5 h HPC infusion
	38.5-79.5 h air at 5 ℓ/min

After the fermentation cycle had progressed 29 hours, some mycelial growth was visible and the pH of the fermentor contents, which had dropped from 5.5 to 4.8, was increased to 5.3 with concentrated NH_4OH (5 ml). At 29.5 hours, the air was turned off and 100 ml of the catalase infusion solution (2% dilution of commercial solution) were added to the fermentor to prevent an early accumulation of toxic concentrations of H_2O_2. The infusion pump was started and catalase and H_2O_2 (8.7%) solutions were delivered via separate channels to the fermentation for the next nine hours. The infusion rate (0.6 ml/min) of the HPC solutions was equivalent to an oxygen supply of 0.08 mM O_2/litre-min. Air was re-introduced to the fermentation at 5 litres/min when the infusion pump was turned off.

It was evident that Aspergillus oryzae continued to grow during the infusion period. Samples taken at the start of the HPC infusion and 4.75 hours later showed an increase in biomass from 0.04 to 0.22% - an average growth rate of approximately 0.36 h^{-1}. Many samples of the fermentor contents were taken during the infusion period and each gave a negative test for free H_2O_2. At 79.5 hours, analysis of the fermentation broth indicated pH of 6.1 and a protease activity of zero. Unfortunately, the frequency of samples during the infusion period had reduced the fermentor contents to an unsatisfactory low level below the upper turbine impeller. As a consequence, the stirred fermentation was terminated, but not before a

shake flask sample was transferred to the shake table. After 22 hours on the shake table, the pH increased to 6.6 and a protease activity of 182 mU/ml was registered. This is a typical level of protease activity from a nitrate-free fermentation medium.

The results of this experiment were encouraging and though the stirred fermentation was discontinued prematurely, it was apparent that the HPC oxygenation system was capable of sustaining cell growth and of maintaining the integrity of the cell to produce protease.

6.15 The Examination of Phosphate Buffer as an Alternative for Nitrate in the Fermentation Medium

If the role of KNO_3 in the fermentation medium were primarily one of pH stabilization at or near 6.5 during the latter portion of the fermentation cycle, then another salt, or combination of salts, with buffer capacity in the pH 6-7 range, might act as a satisfactory replacement. Since KH_2PO_4 (1.37%) was a normal ingredient in the fermentation medium, the addition of some K_2HPO_4 was an obvious choice for an initial examination of a buffer system to restore protease productivity.

Four sets of shake flask fermentations were carried out at 33°C in which KNO_3 (2000 ppm) and K_2HPO_4 (1000 ppm) were introduced into nitrate-free fermentation medium according to

the scheme delineated in Table 40. The table also includes the 95-hour protease titre, the initial and final pH of one flask from each set.

Table 40:

The fermentation response to various combinations of nitrate and dibasic phosphate in the culture medium

KNO_3 ppm	K_2HPO_4 ppm	pH initial	pH final	Protease titre, mU/ml
2000	1000	5.80	6.70	578
0	1000	5.80	6.15	279
2000	0	5.40	6.85	505
0	0	5.40	5.95	156

Though the presence of K_2HPO_4 in the nitrate-free medium was helpful in improving the yield of protease, it was apparent that the amount of K_2HPO_4, or the ratio of KH_2PO_4 to K_2HPO_4 in the above test, was inadequate to buffer the fermentation.

Some of the replicate shake flask fermentations in each set were sacrificed for pH determinations at different time intervals in the cycle. The composite pH profiles, representative of each of the above four fermentation sets, are shown in Figure 14.

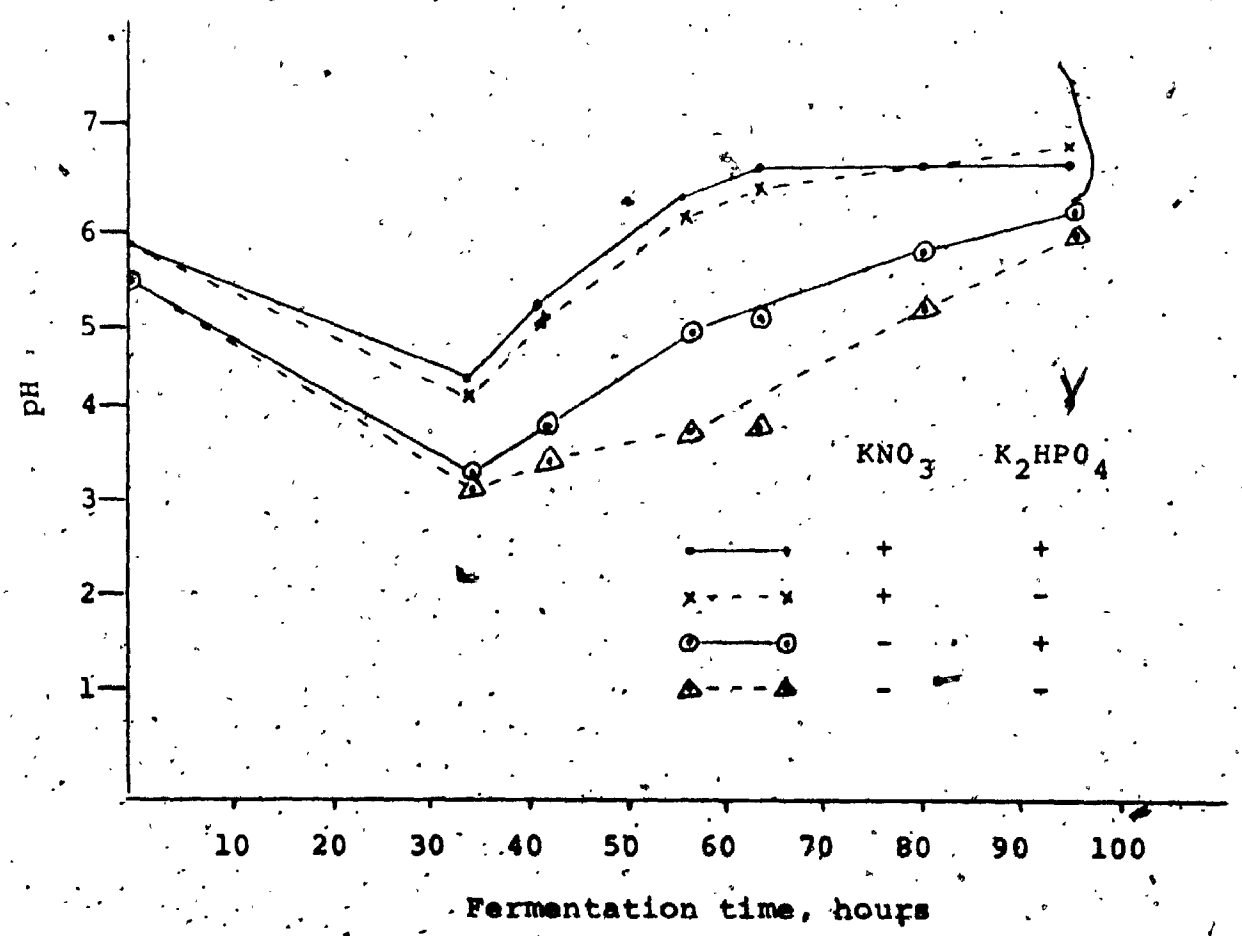


Figure 14. pH profiles of shake flask fermentations using media with various combinations of KNO_3 and K_2HPO_4 at 2000 and 1000 ppm, respectively

It was obvious from the pH data that the presence of K_2HPO_4 did little to alter the final pH of the nitrate-free medium; however, the additional phosphate appeared to increase the rate of recovery from the pH minimum at about 35 hours into the fermentation. The data suggest that the final protease titre (Table 40) is somehow related to the speed that the pH of the fermentation can recover from the low point to a more neutral environment.

6.16 The Response of Aspergillus to Different Phosphate Buffer Systems in lieu of Nitrate in the Fermentation Medium

Though the presence of K_2HPO_4 in nitrate-free media was helpful in improving the yield of protease, it was apparent that the amount of K_2HPO_4, and/or the ratio KH_2PO_4: K_2HPO_4, examined in Section 6.15, was inadequate to buffer the fermentation. A series of shake flask fermentations was set up to examine the fermentation response over a wide range of KH_2PO_4: K_2HPO_4 ratios. The fermentation temperature was 33°C. Table 41 shows the deployment of phosphate in the buffer system with and without nitrate along with the corresponding pH values and protease titres after a 108 hour fermentation cycle.

The results provided evidence that reasonably normal protease titres could be obtained when nitrate was replaced with a suitable buffer system.

Table 41:

The effect of different phosphate buffer systems on the final pH and protease yield of Aspergillus oryzae

Buffer system in media			pH		Protease titre,
KNO_3	KH_2PO_4	K_2HPO_4	initial	final	mU/ml
ppm×10^3	ppm×10^3	ppm×10^3			
0	15	0	5.60	5.90	152
0	14	1	5.90	6.00	230
0	13	2	6.10	6.20	496
0	12	3	6.25	6.30	459
0	11	4	6.40	6.60	545
0	9	6	6.65	6.80	562
2	15	0	5.60	6.70	590
2	14	1	5.85	6.75	574
2	13	2	6.05	6.90	545
2	12	3	6.20	6.95	537
2	11	4	6.35	7.00	677
2	9	6	6.60	7.10	492

6.17 Fourth Assessment of the HPC Technique as a Source of Oxygen for a Fermentation

Having established the fact that the nitrate in the fermentation medium could be replaced by a suitable phosphate buffer system, the HPC technique of oxygenation was re-examined in the stirred fermentor using a nitrate-free medium containing KH_2PO_4 (0.85%) and K_2HPO_4 (0.40%). The fermentation

conditions indicated below were similar to those reported for the experiment described in Section 6.14

volume of nitrate-free medium	12 litres
agitation speed	290 rpm
temperature	32°C
air/HPC regimen	0-25.5 h air at 2 ℓ/min
	25.5-37.5 h HPC infusion
	37.5-87.5 h air at 5 ℓ/min
	87.5-111 h air sparger plugged with mold.

At 25.5 hours into the fermentation cycle, the air was turned off and 100 ml of catalase infusion solution (1.5% dilution of a fresh supply of commercial catalase solution) was added to the fermentor to offset any initial accumulation of peroxide. The infusion pump was started immediately and delivered catalase and H_2O_2 (7.5%) solutions via separate channels to the fermentor during the next twelve hours. The infusion rate of the HPC solutions was set at 1.2 ml/min for the first nine hours and 3 ml/min for the remaining three hours of the twelve hour interval. This was sufficient to supply 0.11 and 0.27 mM O_2/litre-min, respectively, to the culture.

During the infusion period, the pH of the fermentation was increased from 4.1 to 4.7 by the addition of NH_4OH (10 ml). At no time was free H_2O_2 detected in any of the samples of the

fermentor contents. Protease assays at 49, 88 and 111 hours were negative, in spite of the fact that the pH at the end of the fermentation was stabilized in the 6.4-6.6 range.

The zero protease response was somewhat disconcerting in the light of prior shake flask and stirred fermentor experience. There was the possibility that the fermentation was terminated prematurely, however, the most reasonable explanations of the results pointed to:

(i) the lack of sufficient air to the fermentation during the latter part of the cycle when the sparger nozzle became plugged with mycelia

(ii) the toxic accumulation of $C0_2$ in the fermentor during the infusion period and/or when the sparge nozzle was plugged.

To prevent plugging of the sparge nozzle with mycelia and to provide some ventilation for $C0_2$ removal, it seemed prudent to introduce a small but steady air flow to the fermentor contents during all subsequent HPC trial fermentations.

6.18 The Examination of other Nitrogen Sources as a Substitute for Nitrate

Before continuing the stirred fermentation experiments, it was considered useful to examine other sources of nitrogen as a potential replacement for nitrate in spite of the fact that a phosphate buffer system seemed to be satisfactory.

A series of shake flask fermentations, in triplicate, was effected in which ammonium salts and several complex nitrogen sources were tested. Table 42 below details the different treatments of the nitrate-free medium and the pH and protease activity that were achieved.

Table 42:

The pH and protease titres of shake flask fermentations in which nitrate was replaced with other nitrogen sources

Treatment of nitrate-free medium	pH		Protease titre, mU/ml		
	initial	104 h	99 h	104 h	107 h
Control, no KNO_3	5.30	6.05	201	254	202
KNO_3, 2000 ppm	5.35	6.75	690	632	620
Yeast nitrogen base (Difco), 2000 ppm	5.40	5.95	62	66	62
Peptonized milk (Difco), 2000 ppm	5.45	6.15	197	144	149
Bactopeptone (Difco), 2000 ppm	5.45	6.40	316	410	372
Ammonium succinate (monobasic), 2000 ppm	5.50	6.50	492	603	544
$NH_4H_2PO_4$, 2000 ppm + K_2HPO_4, 2000 ppm	5.80	6.10	344	381	339
$NH_4H_2PO_4$, 2000 ppm + K_2HPO_4, 6000 ppm	6.22	6.30	480	406	475

It was apparent from the protease activities of the above shake flask fermentations that complex sources of nitrogen are not required and this effect was supported by the results of the investigation of a possible inductive response (see Section 4.8) when gelatine and peptonized milk were added to standard fermentation medium.

Of the different treatments, the ammonium succinate showed promise as a satisfactory replacement for KNO_3. It was of interest to note that the medium containing ammonium succinate in lieu of nitrate achieved a final pH of 6.5 in spite of the fact that no K_2HPO_4 was added to buffer the system. Obviously, the role of ammonium succinate is more complex than simply providing a source of ammonium nitrogen. The flasks containing ammonium phosphate produced protease titres more in line with those obtained through the introduction of potassium phosphate buffer systems (see Section 6.16).

6.19 Formulation and Testing of a Nitrate-Free Standard Fermentation Medium

The information acquired from the several fermentation experiments examining the role of nitrate was used to formulate (Table 43) a standard nitrate-free fermentation medium.

Table 43:

Standard nitrate-free fermentation medium

sucrose	15 grams/litre	
glucose	7.5 "	
KH_2PO_4	13.7 "	
K_2HPO_4	8.0 "	
$NH_4H_2PO_4$	2.0 "	
NH_4 succinate (monobasic)	2.0 "	
lactic acid	3.54 "	(neutralized with NH_4OH)
$ZnSO_4.7H_2O$	2 ppm	
$FeSO_4.7H_2O$	10 ppm	
$MnSO_4.H_2O$	10 ppm	
Hodag M-8	500 ppm	
pH	6.1	

The above medium was tested simultaneously in the stirred fermentor and in shake flasks. The temperature of both fermentations was 32°C. The agitation and air-sparge rate in the stirred fermentor was 412 rpm and 7 litres/min, respectively. The resultant pH levels and protease titres are given in Table 44.

Table 44:

The response of the standard nitrate-free medium in shake flask and stirred fermentations.

Sample	Fermentation time, hour	pH	Protease titre, mU/ml
shake flask #1	95	6.45	369
#2	95	6.35	545
#3	95	6.50	410
#4	95	6.45	283
#5	110	6.50	590
#6	110	6.55	460
#7	110	6.40	405
#8	110	6.50	435
stirred fermentor	95	6.40	316
	110	6.50	570
	130	6.55	627

Unfortunately, there were no shake flask controls with medium containing nitrate, however, the protease levels reached with the above nitrate-free formulation were typical of those obtained with the former fermentation medium. The above results indicated that it was possible to obtain enzyme activities in stirred fermentations that were equal to, or better than, those in shake flasks when a more vigorous aeration-agitation regimen was utilized.

On the basis of the fermentation response, the nitrate-free formulation (Table 43) with ammonium phosphate and ammonium succinate as nitrogen sources was accepted as the standard fermentation medium for future experiments.

6.20 Fifth Assessment of the HPC Technique as a Source of Oxygen for a Fermentation

This was the initial assessment of the HPC oxygenation technique using the standard nitrate-free medium detailed in the previous experiment. The operational conditions of the stirred fermentor were as follows:

volume of new standard medium	14 litres
agitation speed	400 rpm
temperature	32°C
air/HPC regimen	0-39.5 h air at 7 l/min
	39.5-47.5 h HPC infusion
	47.5-118 h air at 5 l/min

At 39.5 hours into the fermentation cycle, the air to the sparger was reduced from 7 to approximately 0.5 litres/min to prevent the sparger nozzle from plugging with mycelia and to provide ventilation for CO_2 removal from the fermentor. As in the previous attempts at using the HPC technique, an initial dose of catalase infusion solution (60 ml of a 2% dilution of stock commercial catalase solution) was added to the fermentor to prevent accumulation of peroxide. The infusion pump was

started immediately and catalase and H_2O_2, (6.8%) solutions were pumped separately into the fermentor at the rate of 1.2 ml/min. This was equivalent to the addition of oxygen at the rate of 0.09 mM O_2/litre-min.

During the infusion period, the mycelial solids increased from 0.70 to 0.90% (dry basis) and the pH increased from 4.3 to 4.6. At no time was any free H_2O_2 detected in the fermentor contents.

To provide an internal control on the stirred fermentation, four shake flask samples of the fermentor contents were withdrawn immediately before the HPC infusion pump was started. These samples were transferred to the shake table for completion of the fermentation cycle. The resultant pH and protease titres of these four shake flask samples and the stirred fermentation are shown below in Table 44.

Table 44:

The pH and protease titres of the stirred fermentor using the HPC oxygenation technique for eight hours compared to shake flask control samples previously withdrawn from the fermentor

Sample	Fermentation time, hours	pH	Protease titre, mU/ml
shake flask	86	5.5	0
stirred fermentor	86	6.3	246
shake flask	105	6.6	127
stirred fermentor	105	6.8	410
shake flask	118	6.6	286
stirred fermentor	118	6.7	508
shake flask	144	6.6	185

This was the first positive response in a stirred fermentation utilizing the HPC oxygenation technique for a portion of the fermentation cycle. Although the HPC infusion solutions supplied oxygen at a relatively low rate for a period of eight hours, the excellent protease titre that was reached after 118 hours clearly established the efficacy of the HPC technique. The protease titres in the shake flask samples withdrawn from the fermentor were surprisingly low. Large sample volumes in the shake flasks (approximately 75 ml in lieu of 50 ml) may have reduced the degree of aeration and the concomitant protease response of Aspergillus oryzae.

6.21 Sixth Assessment of the HPC Technique as a Source of Oxygen for a Fermentation

This experiment was essentially a repeat of the previous fermentation with some differences as noted. The operational conditions of the stirred fermentor were as follows:

volume of new standard medium	14 litres
agitation speed	400 rpm
temperature	32°C
air/HPC regimen	0-24 h air at 5 ℓ/min
	24-48 h HPC infusion
	48-134 h air at 5 ℓ/min

During the introduction of the HPC solutions to the fermentor, air was sparged at a reduced rate of 0.5 litres/min to provide for ventilation and to avoid plugging of the nozzle. No initial dose of catalase infusion solution was added to the fermentor as it was felt that the possibility of peroxide accumulation was minimal. The HPC solutions (catalase 2% dilution of the stock commercial solution, and H_2O_2 7.7%) were metered into the fermentor to supply oxygen at 0.05 mM O_2/litre-min for the first twelve hours and at 0.1 mM O_2/litre-min for the remaining twelve hours of the infusion period.

Shake flask control samples, withdrawn from the stirred fermentor immediately after inoculation with Aspergillus spores, were fermented on the shaker. It was apparent that the spore density of the inoculated medium was much lower than

normal because the original seed suspension was prepared from premature cultures on the sporulation agar. To examine the effect of this very low seeding rate, additional shake flask samples were fermented with the same medium inoculated with a spore density seven times the concentration of their sister flasks and the stirred fermentor. Pertinent results of the various fermentations are presented in Table 45 below.

Table 45:

Relevant pH, biomass and protease titres of the stirred fermentor using the IIRC oxygenation technique for 24 hours with results from appropriate shake flask controls

Sample	Fermentation time, hours	Biomass	pH	Protease titre, mU/ml
stirred fermentor	24	0.20	5.65	-
stirred fermentor	35	0.35	4.20	-
stirred fermentor	46	0.75	4.05	-
stirred fermentor	48	0.82	4.30	-
stirred fermentor	59	-	5.00	-
stirred fermentor	82	-	6.00	-
stirred fermentor	91	-	6.40	102
shake flask control	91	-	5.80	0
shake flask (7 x spores)	91	-	5.75	0
stirred fermentor	114	-	6.90	279
shake flask control	114	-	6.15	20
shake flask (7 x spores)	114	-	6.80	525
stirred fermentor	134	-	6.90	344
shake flask control	134	-	6.70	295
shake flask (7 x spores)	134	-	6.75	402

In spite of its low spore inoculum, the stirred fermentor produced higher protease titres in a shorter time than the corresponding shake flask controls. Of special interest was the fact that the biomass in the stirred fermentor increased four-fold during the HPC infusion interval. High spore inocula gave higher yields of protease.

6.22 Determination of the Activity and Stability of Catalase Powder for HPC Applications

As indicated earlier, the source of catalase used in the research work already reported was a technical grade beef liver concentrate which was prone to microbial contamination and loss of activity on storage for several months. It was decided to evaluate a lyophilized preparation of beef liver catalase as a more stable and reliable source of enzyme.

According to the manufacturer (Nutritional Biochemicals Corporation) 1 gram of lyophilized catalase powder should decompose 1200-1400 grams of H_2O_2. Upon testing this powder for enzyme activity in the catalase assay apparatus (Section 3.3.2), 1 gram of the material was able to decompose 1500 grams H_2O_2 in ten minutes at 25°C under the standard test conditions.

The formaldehyde-carrier solution (Section 6.7) containing 250 ppm catalase powder was adopted as the standard infusion solution. The catalase activity of this solution remained constant on storage at room temperature for a test period of

eight days. At identical infusion rates of the catalase and H_2O_2 solutions there was sufficient enzyme activity to ensure destruction of a 3% H_2O_2 infusion solution. Since the strength of the peroxide infusion solution was normally around 10%, there was an adequate excess to accommodate any limitations normally encountered in research.

6.23 Seventh Assessment of the HPC Technique as a Source of Oxygen for a Fermentation

The following experiment was the last and most comprehensive of the HPC oxygenation series. To provide some indication of the dissolved oxygen levels and oxygen demand rates, the oxygen electrode was introduced into the stirred fermentor during the infusion period. Furthermore, a separate stirred fermentation control provided comparative data regarding the specific contribution of the HPC solutions. The operational conditions of the control and HPC fermentations were as follows:

	Control	HPC Fermentation
volume	14 litres	14 litres
agitation	400 rpm	400 rpm
temperature	32°C	32°C
air/HPC regimen	0-24 h air at 5 ℓ/min	0-33.5 h air at 5 ℓ/min
	24-48 h air at 0.5 ℓ/min	33.5-40 h HPC infusion, air at 0.3 ℓ/min
	48-159 h air at 5 ℓ/min	40-127 h air at 5 ℓ/min

In addition to the stirred fermentation control, several samples were removed from the trial fermentation before the introduction of the HPC solutions and fermented to completion on the shake table to provide reference data.

At 33.5 hours into the trial fermentation cycle, the air sparge rate was reduced from 5 to 0.3 litres/min and the HPC infusion pump was started. Catalase (250 ppm of lyophilized powder) and H_2O_2 (10%) were infused into the stirred contents of the fermentor at controlled rates for a 6.5 hour period. Three discrete infusion rates were selected to supply oxygen to the culture of Aspergillus at 0.06, 0.13, and 0.32 mM O_2/litre-min. These rates were deliberately varied to check the potential for feed-back control to stabilize the dissolved oxygen level at any pre-determined value. Figure 15 represents the actual dissolved oxygen value in response to step-wise variations in the infusion feed rates. The response was reasonably rapid and leaves little doubt that a simple control loop consisting of an oxygen electrode and a dissolved oxygen recorder-controller to modulate the speed of the HPC infusion pumps could program the dissolved oxygen concentration at any practical level.

Assuming an efficiency of the HPC oxygenation technique close to 100% - and evidence is presented in the next chapter of this thesis to support the contention - it becomes a simple matter to estimate the oxygen demand of the culture at any time during the infusion period. If the dissolved oxygen concentration is maintained at a constant value for a discrete

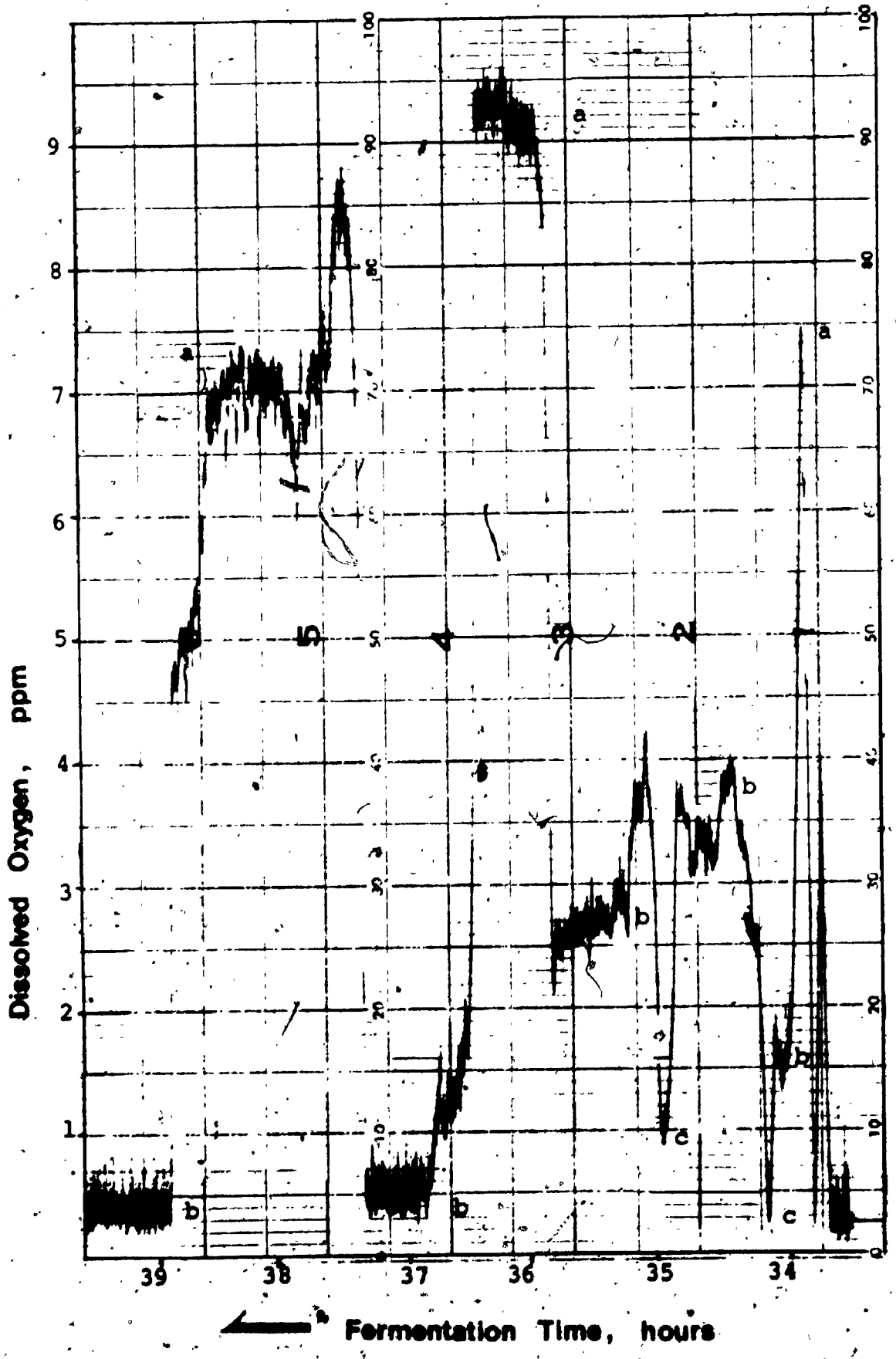


Figure 15. Dissolved oxygen read-out during HPC infusion.

Infusion rates: a = 3.0 ml/min = 0.32 mM O_2/l-min,
b = 1.2 ml/min = 0.13 mM O_2/l-min
c = 0.6 ml/min = 0.06 mM O_2/l-min

time interval, the H_2O_2 added to the fermentor can be equated to the biological oxygen demand. This technique would be equally applicable to dissolved oxygen concentrations above and below the critical value for the culture under consideration. For example, Figure 15 indicates that at approximately 38 hours into the fermentation cycle, the dissolved oxygen level remained reasonably constant for thirty minutes. By equating the oxygen supply to the oxygen demand during this time interval, it follows from the strength and infusion rate of the peroxide solution that the oxygen demand was equal to 0.32 mM O_2/litre-min. Since the biomass at this point in time was 0.70%, the specific demand of the Aspergillus oryzae culture was 0.046 mM O_2/gram-min.

Figures 16 and 17 show the growth, pH and product formation responses for the control and HPC fermentations, respectively. Both experiments gave the typical pH and protease activity responses during the fermentation. Significantly, the average growth rate of the culture during the first 4.5 hours of the HPC infusion period was 0.10 h^{-1}, whereas the average growth rate of the control fermentation during a similar time interval at the reduced air rate of 0.5 ℓ/min was 0.02 h^{-1}.

The protease titre peaked at 520 mU/ml in 6.5 days for the control and peaked at 373 mU/ml in 4.5 days for the HPC fermentation. In spite of the fact that the cycle of the HPC fermentation was shortened by approximately two days, the protease titres were disappointingly low; nevertheless, they were higher than the control shake flask samples taken from the

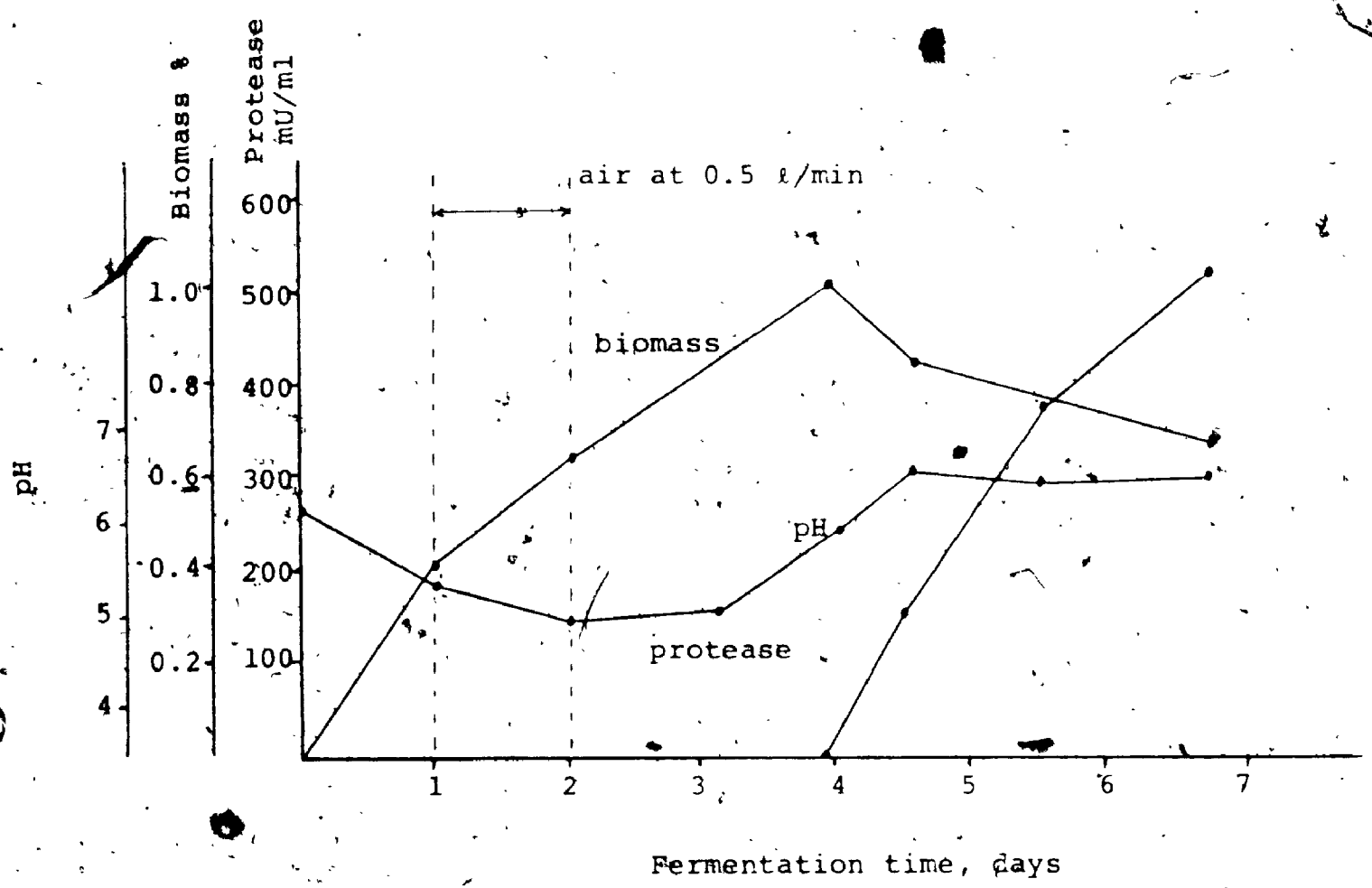


Figure 16. Stirred fermentation control, air at 5 ℓ/min except as indicated

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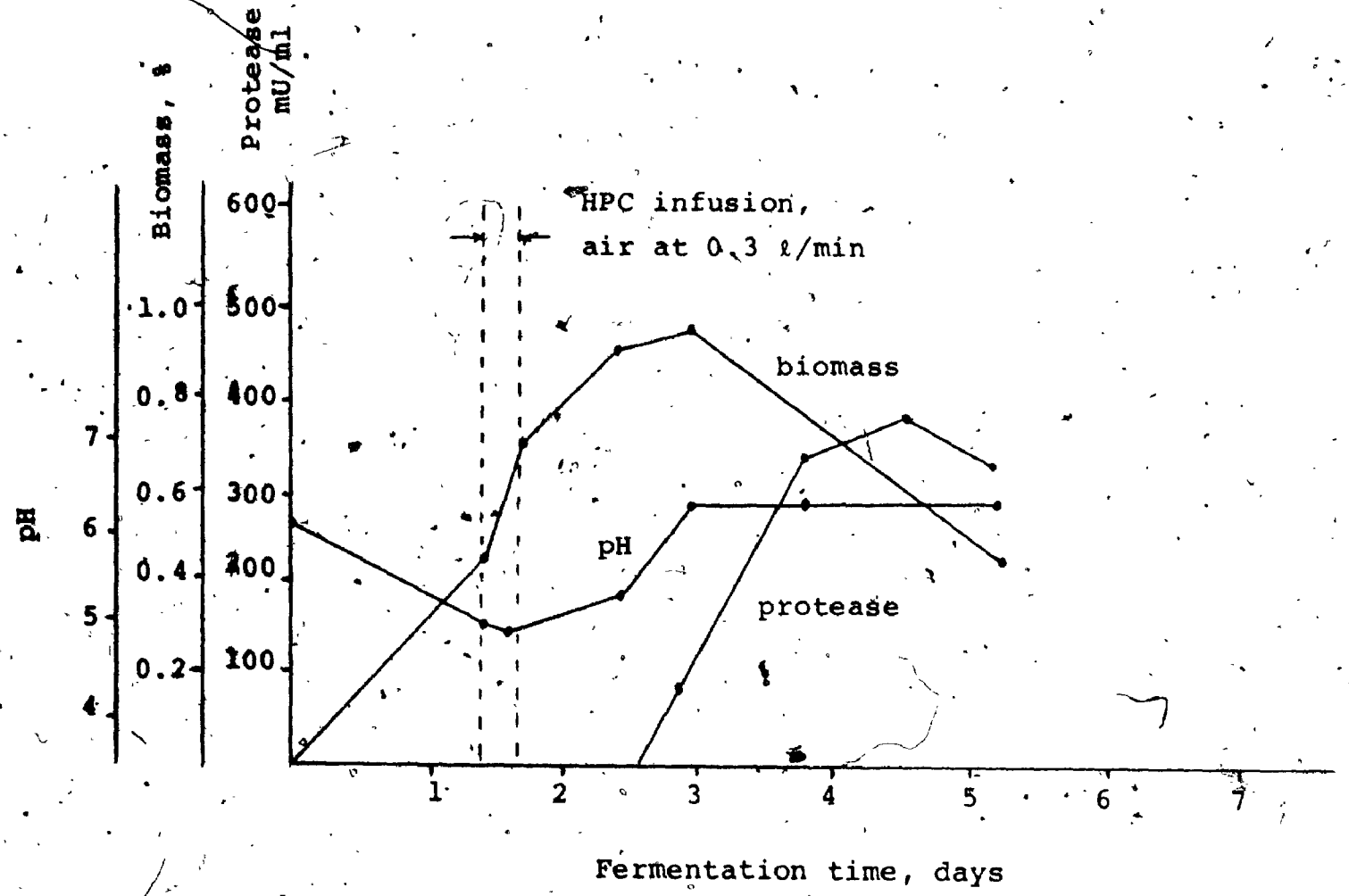


Figure 17. HPC fermentation, air at 5 ℓ/min except as indicated

fermentor at the time of seeding. The shake flask samples produced a maximum protease titre of 242 mU/ml in 6.5 days suggesting that the spore density of the original seeding was inadequate - as was demonstrated in the experiment outlined in Section 6.21.

Photographs (Figures 18, 19, 20) show the stirred fermentor with ancillary equipment during the HPC oxygenation of the Aspergillus oryzae fermentation.

Figure 18. Fermentation Design 14-litre bench fermentor with HPC infusion pump assembly

Figure 19. Head assembly of 14-litre bench fermentor

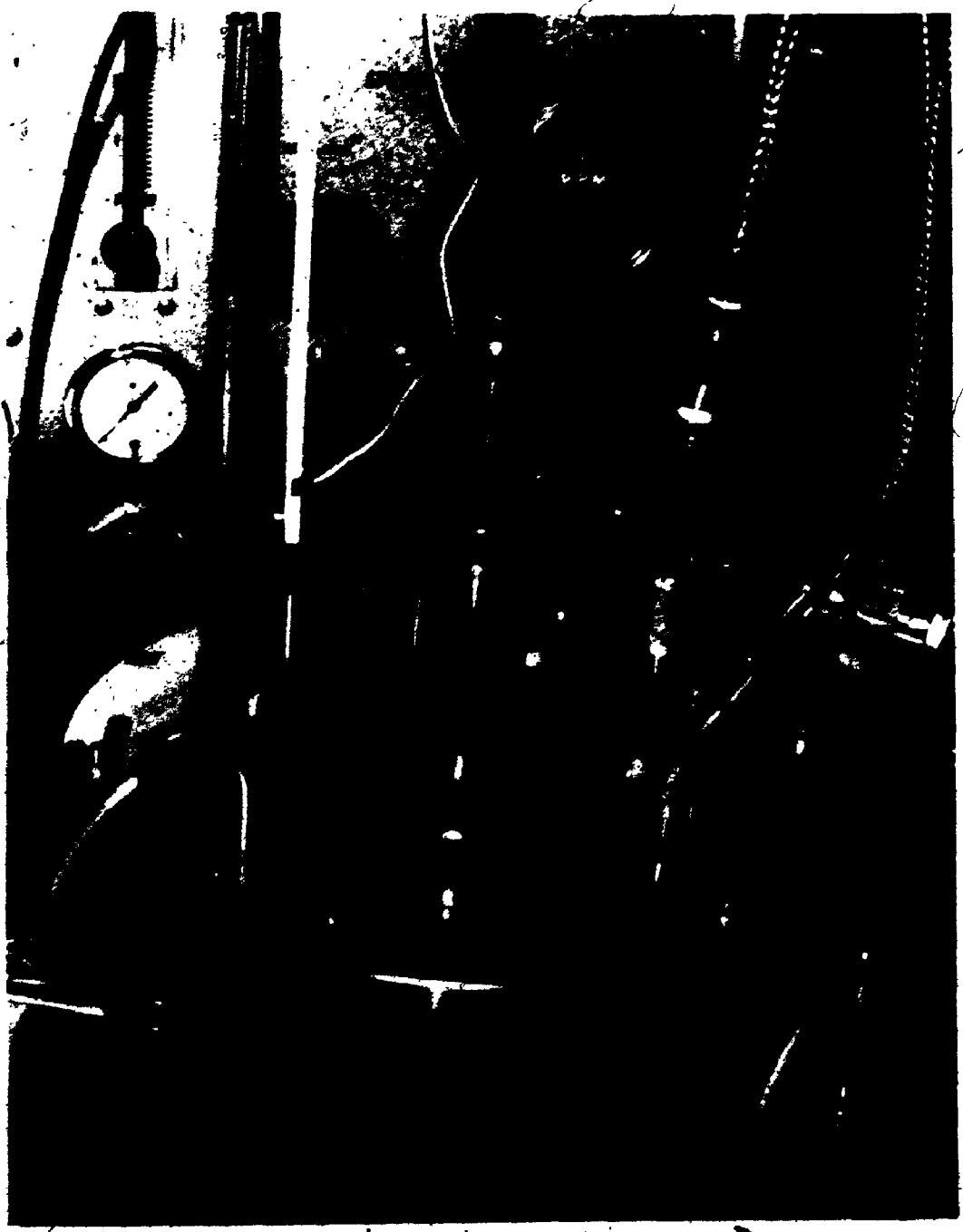
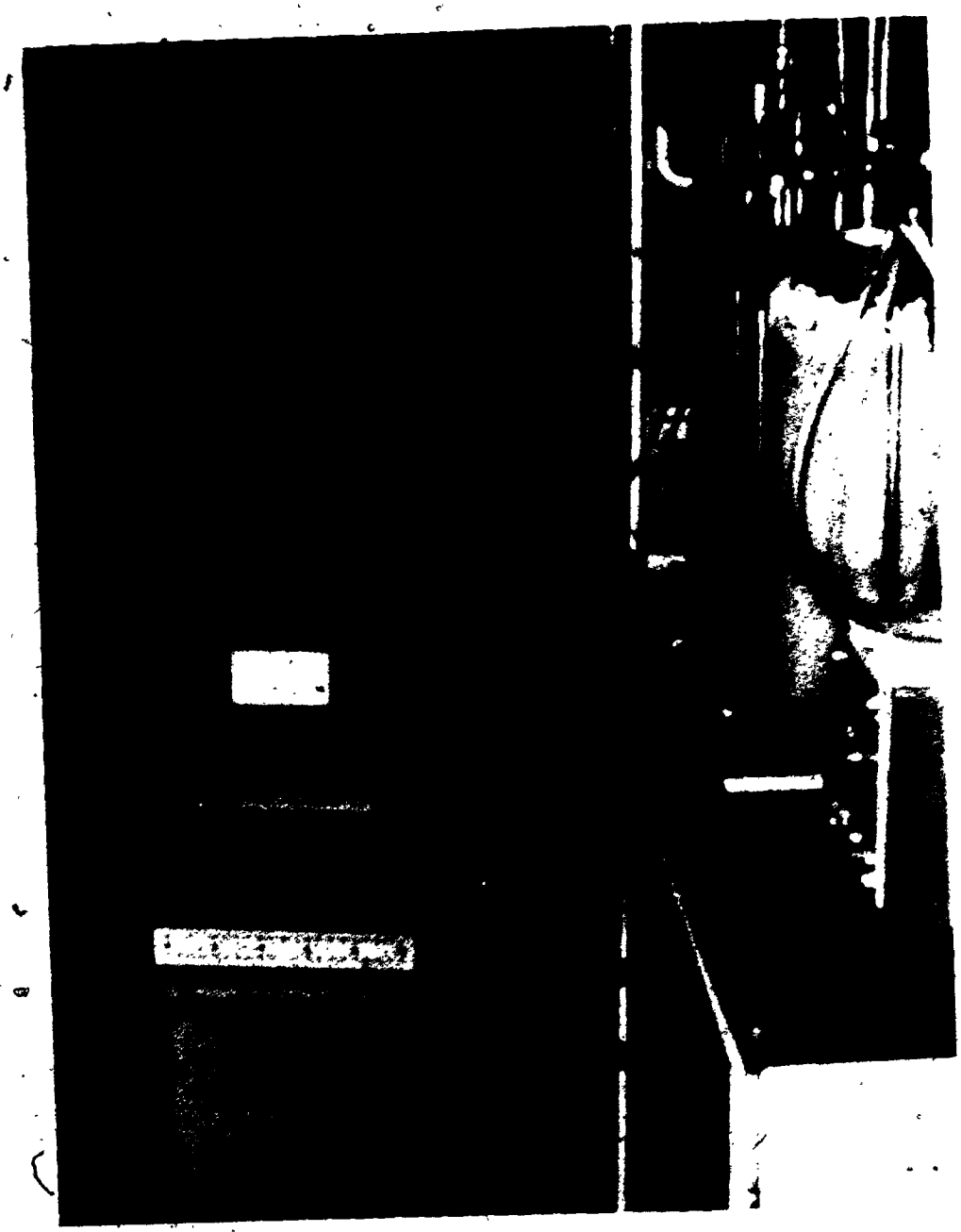


Figure 20: Beckman oxygen analyser (model 778) with Kent Multelec MK III strip chart recorder



CHAPTER 7: GLUCONIC ACID PRODUCTION AS A MEASURE OF THE EFFICIENCY OF THE HPC OXYGENATION TECHNIQUE

The research emphasis reported in this thesis has been directed towards the development of a practical technique for the enzymatic release of oxygen in a non-Newtonian Aspergillus oryzae fermentation during the period of peak oxygen demand. If it can be shown that the efficiency of the HPC technique, as a source of available oxygen for biological processes, is high, then the possibility of limited application in the fermentation industry becomes promising.

The sulfite oxidation studies (Section 5.1) demonstrated that oxygen transport was reduced to about 25% of its previous value when paper pulp solids were introduced into the liquid phase to simulate mycelia. This reduction in oxygen transport in non-Newtonian systems cannot always be compensated because of the physical limitations of the agitation-aeration equipment of the fermentor.

Ideally, the efficiency of oxygen transport should be measured under actual fermentation conditions. The dynamic technique, suggested by Taguchi and Humphrey (1966) could have been used to measure the efficiency of the HPC oxygenation system, but it would have involved lengthy and difficult

fermentation procedures. It was decided to avoid the active fermentation approach in favor of a model fermentation system. This model consisted of glucose and glucose oxidase which was used to simulate the oxygen fixation mechanism of the cell and paper pulp to represent the physical presence of mycelia. Hsieh (1969) described the conversion of glucose to gluconic acid in the presence of glucose oxidase as a useful index of oxygen transport. The rate of accumulation of gluconic acid, as determined by a simple acid-base titration, can be used as a direct measure of the mass transfer of oxygen to the liquid phase.

Before undertaking specific oxygen transport studies, several preliminary experiments were run to determine the compatibility of paper pulp, glucose oxidase, glucose, catalase, H_2O_2, and formaldehyde when present simultaneously in an aqueous milieu.

7.1 The Effect of Asbestos on Activity of Glucose Oxidase

It was known that some grades of filter paper that were available as a source of paper pulp contained a small percentage of asbestos fibres and it was considered important to determine if this material was inhibitory to the activity of glucose oxidase.

The materials were evaluated on a reciprocal shaker in 300 ml conical flasks. Glucose monohydrate (2%) was made up in a weakly buffered phosphate solution at pH 6.4 (0.15% KH_2PO_4 and

0.1% K_2HPO_4) and 50 ml aliquots of this substrate solution were added to each of several flasks. The appropriate fibrous material (1%) and glucose oxidase* (2000 ppm of the commercial solution) were introduced into the glucose solution and the reaction was permitted to proceed for 105 minutes at 26°C. The gluconic acid (G.A.) generated and the corresponding oxygen transfer rates are shown in Table 46.

Table 46:

The inhibitory effect of asbestos on glucose oxidase production in shake flasks after 105 minutes at 26°C

Treatment	mM G.A. per flask	OAR**, mM O_2/ℓ-min
Control, no solids	3.5	0.33
Carlson E K filter paper, 1%, contains asbestos fibres	0.4	0.04
Pure asbestos fibres, 1%	0.4	0.04
Cellulose paper pulp, 1%	2.9	0.28

**Oxygen absorption rate

There was no doubt that asbestos, even in relatively small amounts, interfered with the enzymatic oxidation of glucose. Cellulose paper pulp, on the other hand, was non-toxic, after due allowance for reduced oxygen transport in the presence of suspended solids.

*Dee O, supplied by Miles Laboratories, Elkhart, Indiana.

7.2 The Effect of HPC Infusion Solutions on the Activity of Glucose Oxidase

An initial test in the stirred fermentor, in the presence of 1% paper pulp, indicated a steady production of gluconic acid when air was sparged into the medium, however, when the HPC solutions were substituted for the air the production of gluconic acid stopped within fifteen minutes and would not respond to the subsequent introduction of air. Formaldehyde, present in the catalase carrier solution, was immediately suspected as being the toxic agent. Two separate shake flask studies, under conditions similar to those in the previous experiment, were carried out to investigate more fully the nature of this toxicity. Varying amounts of formaldehyde, H_2O_2, paper pulp and asbestos were introduced into shake flasks containing 50 ml of buffered glucose substrate (glucose monohydrate 2%) and glucose oxidase (2000 ppm of Dee-O). The gluconic acid produced in each set of flasks was determined at the end of 75 and 90 minutes, respectively. The results are shown in Tables 47 and 48.

Table 47:

The inhibitory effect of HPC infusion solutions and formaldehyde on the glucose - glucose oxidase system. Gluconic acid was measured after 75 minutes on the shake table at 26°C

Treatment	mM G.A. per flask	OAR mM O_2/ℓ-min
Control	2.5	0.33
H_2O_2, 20 ppm	2.6	0.35
H_2O_2, 200 ppm	2.6	0.35
Catalase infusion solution, to give formaldehyde 30 ppm	0.5	0.07
Formaldehyde, 1500 ppm	0	0
Formaldehyde, 7500 ppm	0	0

Table 48:

The inhibitory effect of asbestos pulp and formaldehyde on the glucose - glucose oxidase system. The flasks were on the shake table for ninety minutes at 25°C

Treatment	mM G.A. per flask	OAR mM O_2/ℓ-min
Control	2.9	0.32
Paper pulp, 2%	1.8	0.20
Asbestos pulp, 2%	0.3	0.03
Formaldehyde, 7 ppm	1.2	0.13
Formaldehyde, 15 ppm	0.7	0.08
Formaldehyde, 37 ppm	0.3	0.03
Formaldehyde, 74 ppm	0.1	0.01

The somewhat lower amount of gluconic acid produced in the flask containing 2% paper pulp, could be accounted for in terms of physical impairment of oxygen transport. However, the more severe reduction in the amount of gluconic acid produced in the presence of asbestos and formaldehyde indicated that both those agents were inhibitory to the glucose - glucose oxidase system.

7.3 The Estimation of Catalase Activity in the Glucose Oxidase Solution

During the oxygenation studies in the stirred fermentor, it was observed that H_2O_2 in the presence of glucose and glucose oxidase solution produced some gluconic acid, whereas H_2O_2 and glucose alone did not react in any detectable way. It seemed reasonable to suspect that the commercial glucose oxidase preparation contained appreciable amounts of catalase activity.

Utilizing the assay procedure described in Section 3.3.2 the catalase activity of the glucose oxidase solution was measured. The analysis indicated that the catalase activity of the glucose oxidase solution was 5% that of the catalase powder on a weight for weight basis.

It was reasoned that the catalase activity in the commercial oxidase solution would not seriously interfere with the oxygenatic studies provided that the overall catalase activity of the system was not rate limiting.

7.4 The Measurement of the Efficiency of the HPC Oxygenation Technique

The production of gluconic acid in a defined glucose - glucose oxidase system, was used to measure the oxygen transport efficiency of the HPC oxygenation technique.

Three series of tests were carried out in the stirred fermentor. The reaction volume in each case was 12 litres and the temperature was maintained at 31°C. The substrate concentration was 2% glucose monohydrate weakly buffered with phosphate to pH 6.5. Suspended solids, when present, consisted of a 1% suspension of ground paper pulp prepared in the Waring-type blendor. Glucose oxidase solution was added to the fermentor contents to give a 0.1% dilution of the stock commercial solution.

The three levels of agitation investigated were 200, 400, and 600 rpm and the aeration levels selected for each series were 0, 0.5, 2, and 4 litres/min. The catalase solution (700 ppm as the lyophilized powder) and the H_2O_2 solution (10%) were infused through separate channels to the fermentor at three discrete rates: 0.6, 1.2, and 3 ml/min. The HPC oxygenation efficiency was tested only in the presence of suspended solids as this represented the more challenging set of test conditions.

A drop in the pH of the stirred fermentor contents indicated the production of gluconic acid. Standard sodium

hydroxide was added at frequent intervals to restore the pH to the original level of 6.5. The pH was not permitted to drop below 5.5 at any time during the thirty-minute reaction period for each set of test conditions. The accumulation of gluconic acid in milli-moles and the associated oxygen transfer rate were calculated from the total volume of sodium hydroxide required to restore the pH to its original value on completion of the thirty-minute reaction period. For any particular level of agitation, some oxygen was unavoidably picked up from the head space air giving a corresponding increase in gluconic acid production. The gluconic acid generated by surface aeration was subtracted from the total acid production to get the net product formation for each set of conditions. Sufficient glucose was present as substrate in the fermentor to provide a maximum gluconic acid concentration of 100 milli-moles/litre (1200 milli-moles in the fermentor).

The efficiency of aeration was calculated by dividing the milli-moles of oxygen required to produce the gluconic acid by the milli-moles of oxygen entering the fermentor as air through the sparger. In a like manner, the efficiency of the HPC technique was calculated by dividing the milli-moles of oxygen required to produce the gluconic acid by the milli-moles of available oxygen in the H_2O_2 infusion stream. Differences in the reported values for the available oxygen originating from the metered H_2O_2 infusion stream reflect concentration

variances in solution make-up. Gluconic acid production and the corresponding oxygenation efficiencies are shown in Tables 49, 50 and 51.

Table 49:

Gluconic acid production as a measure of the oxygenation efficiences of conventional aeration and HPC techniques. The volume in the stirred fermentor was 12 litres, agitation speed and temperature were 200 rpm and 31°C, respectively

Paper pulp, %	Oxygen Supply		G.A. Production*		OAR**	Oxygenation efficiency, %
	ℓ air per min	mM 0_2 per ℓ-min	surface aeration	sparger aeration	mM 0_2 per ℓ-min	
0	0	0	6.7	0	0	-
0	0.5	0.38	6.7	14.3	0.020	5.2
0	2.0	1.54	6.7	45.8	0.064	4.2
0	4.0	3.08	6.7	75.4	0.105	3.4
1	0	0	2.9	0	0	-
1	0.5	0.38	2.9	12.4	0.017	4.4
1	2.0	1.54	2.9	32.4	0.045	2.9
1	4.0	3.08	2.9	37.2	0.052	1.7

Paper pulp, %	Oxygen Supply		G.A. Production		OAR	Oxygenation efficiency, %
	HPC ml/min	mM 0_2 per ℓ-min	surface aeration	infusion	mM 0_2 per ℓ-min	
1	0	0	2.9	0	0	-
1	0.6	0.067	2.9	49.6	0.069	103
1	1.2	0.132	2.9	92.6	0.129	98
1	3.0	0.331	2.9	217.1	0.302	91

* gluconic acid production in milli-moles during thirty-minute interval.

** oxygen absorption rate

Table 50:

Gluconic acid production as a measure of the oxygenation efficiencies of conventional aeration and HPC techniques. The volume in the stirred fermentor was 12 litres, agitation speed and temperature were 400 rpm and 31°C, respectively

Paper pulp, %	Oxygen Supply		G.A. Production*		OAR** mMO_2 per ℓ-min	Oxygenation efficiency, %
	ℓ air per min	mM O_2 per ℓ-min	surface aeration	sparger aeration		
0	0	0	28.6	0	0	-
0	0.5	0.38	28.6	57.3	0.080	20.8
0	2.0	1.54	28.6	105.1	0.146	9.5
0	4.0	3.08	28.6	124.2	0.172	5.6
1	0	0	14.3	0	0	-
1	0.5	0.38	14.3	23.9	0.033	8.6
1	2.0	1.54	14.3	43.0	0.060	3.9
1	4.0	3.08	14.3	71.6	0.099	3.2

Paper pulp, %	Oxygen Supply		G.A. Production		OAR mMO_2 per ℓ-min	Oxygenation efficiency, %
	HPC ml/min	mM O_2 per ℓ-min	surface aeration	infusion		
1	0	0	16.2	0	0	-
1	0.6	0.073	16.2	50.6	0.070	96
1	1.2	0.146	16.2	98.4	0.137	94
1	3.0	0.364	16.2	203.4	0.282	77

* gluconic acid production in milli-moles during thirty-minute interval

** oxygen absorption rate

Table 51:

Gluconic acid production as a measure of the oxygenation efficiencies of conventional aeration and HPC techniques. The volume in the stirred fermentor was 12 litres, agitation speed and temperature were 600 rpm and 31°C, respectively

Paper pulp, %	Oxygen Supply		G.A. Production*		OAR**	Oxygenation efficiency, %
	ℓ air per min	mM O_2 per ℓ-min	surface aeration	sparger aeration	mMO_2 per ℓ-min	
0	0	0	35.3	0	0	-
0	0.5	0.38	35.3	63.1	0.088	22.9
0	2.0	1.54	35.3	139.5	0.194	12.6
0	4.0	3.08	35.3	148.1	0.206	6.7
1	0	0	16.2	0	0	-
1	0.5	0.38	16.2	24.9	0.035	9.1
1	2.0	1.54	16.2	60.2	0.084	5.4
1	4.0	3.08	16.2	86.0	0.119	3.9

Paper pulp %	Oxygen Supply		G.A. Production		OAR mMO_2 per ℓ-min	Oxygenation efficiency, %
	HPC ml/min	mM O_2 per ℓ-min	surface aeration	infusion		
1	0	0	9.6	0	0	-
1	3.0	0.375	9.6	219.6	0.305	81

* gluconic acid production in milli-moles during thirty-minute interval

** oxygen absorption rate

The sets of data in the above tables suggest greater precision and accuracy than, in fact, could be realized. Physical limitations in the control and measurement of the different agitation speeds, aeration and infusion rates, kinetic effects arising from the progressive depletion of glucose substrate during the test, and the inadequacies of the analytical procedure for gluconic acid estimation together contributed to what was undoubtedly a large standard error in the results. Notwithstanding, these compromising factors, the data demonstrated that the HPC oxygenation technique in the presence of suspended solids was markedly more efficient than the conventional aeration technique. The oxygen transfer efficiency of the HPC technique in the presence of suspended solids remained uniformly high - approaching 100% in certain instances and greater than 75% in all instances.

A recheck of the gluconic acid production when the HPC solutions were infused at 3 ml/min into freshly prepared glucose substrate, stirred at 400 rpm and containing suspended solids, indicated an oxygen transfer efficiency of 90% as compared to 77% in the test reported in Table 50.

The results suggest that the ability of the HPC solutions to supply oxygen is largely independent of agitation power, and by inference, independent of the amount of suspended solids provided that there is sufficient agitation to keep the vessel contents uniformly dispersed.

Unfortunately, the concentration of the paper pulp solids and the agitation-aeration regimens that were selected for the glucose oxidation studies did not match well with those used in the sulfite oxidation studies reported in Section 5.1. Generally the oxygen transfer rates obtained from both the chemical and biological techniques were of the same order of magnitude. In the one or two instances that could be compared, the sulfite oxidation rates were higher than the glucose oxidation rates. For example, in the absence of suspended solids the SOR for an agitation-aeration regimen of 300 rpm and 2 litres air/min was 0.19 mM O_2/litre-min, whereas the OAR for the glucose system was 0.15 mM O_2/litre-min when stirred at 400 rpm and supplied with air at 2 litres/min. The higher SOR rate is almost certainly due to the smaller bubble size induced by the presence of the sulfite salt.

7.5 Power Measurements of the Stirred Fermentor

Having demonstrated the relatively high oxygen transfer efficiencies of the HPC technique, the following experiment was undertaken to measure the power input associated with the specific agitation-aeration regimens used in the oxygenation studies detailed in Section 7.4. The torque meter was adjusted to zero with the stirrer turning at the appropriate speed in the empty fermentor. The torque in inch-pounds and the calculated horse power per 1000 gal for each set of operating conditions, are reported in Table 52 below.

Table 52:

Power absorbed by the contents of the stirred fermentor under different conditions of agitation, aeration and suspended solids. The fermentor volume was 12 litres

Paper pulp, %	Agitator speed / Air, l/min	200 rpm		400 rpm		600 rpm	
		Torque in-lb	HP/1000 gal	Torque in-lb	HP/1000 gal	Torque in-lb	HP/1000 gal
0	0	0.54	0.64	2.24	5.4	4.76	17.1
0	0.5	0.52	0.62	2.20	5.3	4.68	16.9
0	2.0	0.44	0.53	2.06	5.0	4.26	15.4
0	4.0	0.32	0.38	1.60	3.9	3.22	11.6
1	0	0.68	0.82	2.76	6.6	5.64	20.3
1	0.5	0.58	0.70	2.72	6.5	5.50	19.8
1	2.0	0.52	0.62	2.28	5.5	5.08	18.3
1	4.0	0.50	0.60	1.84	4.4	4.18	15.0

For any given increase in the speed of agitation, power input increases as the cube of the speed ratio. The above power measurements are in general agreement with this relationship though some errors were involved in measuring and controlling the speed of agitation. Typically, the power requirement decreased as the gassing rate was increased. The power per unit volume measurements cover the range normally encountered in non-Newtonian fermentations. Steel and Maxon (1966b) reported that 6-10 HP/1000 U.S. gal are required for effective agitation of large scale novobiocin fermentations.

The gluconic acid production data (Section 7.4) and the corresponding agitation power data reported above, demonstrated that the HPC oxygenation technique with an agitation power input less than 1 HP/1000 gal out-performed the air-sparged system operating at 15 HP/1000 gal.

CHAPTER 8: FERMENTOR DESIGN

8.1 Design Problem

The bench-scale fermentation described in this thesis is to be scaled-up to an industrial fermentation facility with an operating capacity of 90.7 m^3 (20,000 gallons). It is desired to specify the dimensional characteristics of the fermentor and agitation system to conform to acceptable engineering criteria for complete mixing, terminal mixing time and impeller tip velocity.

The total energy requirements for agitation and aeration of the fermentation are to be calculated for two separate operational conditions; one as a conventional air-sparged *Aspergillus oryzae* fermentation and the other as the same fermentation utilizing the HPC technique to supply supplemental oxygen to the culture during the peak demand period. Process costs, as they relate to both systems for supplying oxygen to the fermentation, are to be examined.

8.2 Notation

D_T = diameter of fermentor, m

H_L = fluid depth, m

D_i = impeller diameter, m

W = impeller blade width, m

L = impeller blade length, m

B = number of blades on impeller

R = number of baffles

J = baffle width, m

N = impeller rotational speed, rev/s

μ = viscosity, kg/m s

N_{Re} = impeller Reynolds number, dimensionless, $N D_i^2 \rho/\mu$

N_{Re}' = modified impeller Reynolds number, dimensionless

$$\frac{N^{2-n} D_i^2 \rho}{0.1K \left(\frac{6n + 2}{n}\right)^n}$$

I = number of impellers

K = consistency index, kg/m s^{2-n}

n = flow behaviour index

g = gravitational acceleration, m/s^2

g_c = gravitational constant

N_p = Power number, dimensionless, $Pg_c/\rho N^3 D_i^5$

N_t = mixing time factor, dimensionless,

$$t\,(N\,D_i^2)^{2/3}\,g^{1/6}D_i^{1/2}\Big/\,H_L^{1/2}D_T^{3/2}$$

t = mixing time, s

ρ = fluid density of fermentation medium, kg/m^3

P_0 = power absorbed in agitation of ungassed medium, kg m/s, HP

P_g = power absorbed in agitation of gassed medium, kg m/s, HP

k_La = volumetric absorption coefficient, 1/min

Q = air sparge rate, m^3/min

V = operational volume in fermentor, m^3

8.3 Design Assumptions and Criteria

8.3.1 Geometric similarity

Geometric similarity is to be maintained between the bench-scale fermentor and the large scale fermentor. Modifications, as necessary, are to be introduced to conform to engineering design constraints.

8.3.2 Rheological properties of suspended solids

The rheological properties of a paper pulp suspension and those of a mycelial suspension of *Aspergillus* are assumed to be identical. Solids of *Aspergillus* are not expected to exceed 1% (dry basis).

8.3.3 Oxygen transfer rates in the presence of suspended solids

The oxygen transfer rates in the presence of paper pulp solids and mycelial *Aspergillus* are assumed to be identical for identical concentrations. Brierley and Steel (1959) showed that under the same conditions of agitation and air flow, filamentous forms of *Aspergillus niger* and paper pulp, up to 2.5% (dry basis), gave similar values of $k_L a$.

8.3.4 Power per unit volume

Scale-up is to be predicated on a constant power/unit volume basis. Data here have established that oxygen transport in the presence of suspended pulp solids is more responsive to changes in agitation power than to changes in aeration rate. This is supported by the observation (Bierley and Steel, 1959) that the practice of increasing air flow to increase oxygen transport is essentially useless for fungal fermentations.

8.3.5 Modified Reynolds numbers

Modified Reynolds numbers 'N_{Re}' are to be consistent with minimum values expressed by Norwood and Metzner (1960) for complete mixing with a turbine impeller in non-Newtonian systems. N_{Re} should exceed 270 for a $D_T/D_i = 3.0$; 110 for a $D_T/D_i = 2.0$ and 90 for a $D_T/D_i = 1.5$.

8.3.6 Power absorbed per impeller

The power absorbed by individual impellers on the same shaft is taken to be strictly additive (Richards, 1963).

8.3.7 Terminal mixing time

The terminal mixing time 't' for the HPC infusion solutions in the large fermentor should not be greater than 10 times that calculated for the bench fermentor operating at the lower agitation speed. Norwood and Metzner (1960) suggested that mixing time data obtained from Newtonian systems can be applied

to pseudoplastic systems - at least until more reliable data becomes available. The empirical correlation of the mixing time factor 'N_t' with impeller Reynolds number 'N_{Re}' is similar in form to that of Power number 'N_p' versus Reynolds number 'N_{Re}' wherein both N_t and N_p are constant and independent of fluid viscosity in the turbulent region. For a typical fermentor equipped with a single turbine impeller the mixing time factor 'N_t' is approximately equal to 6, although it must be assumed that there is complete mixing of the tank contents within a turbulent flow regime (Aiba et al., 1965).

8.3.8 Liquid depth in fermentor

In the calculation of the terminal mixing time 't' the liquid depth 'H_L' is to be designated as the liquid depth per unit impeller on the agitator shaft.

8.3.9 Fermentation medium

The fermentation medium, that was used in the small scale trials reported herein, is to be used without change in the large scale fermentor.

8.3.10 Fermentation cycles

In an Aspergillus fermentation which utilizes the HPC infusion technique, it is expected that it will be completed in four days during which air is to be sparged at a uniform rate of 0.033 V/V/min. Hydrogen peroxide and catalase are to be infused for one day in response to a dissolved oxygen control system.

The conventional fermentation utilizing air as the sole source of oxygen is expected to require five days for completion. It is assumed that air is to be sparged at the maximum rate of 0.33 V/V/min for two days and at a reduced rate of 0.033 V/V/min for the other three days of the fermentation.

8.3.11 Requirement of HPC materials for cost estimation

For purposes of cost estimation it is assumed that the oxygenation efficiency of the HPC technique is 100% and that this enzymatically released oxygen is supplied at a uniform rate of 0.24 mM O_2/litre-min for a period of one day. The HPC infusion system is to be capable of supplying oxygen to the fermentation over the range of 0.1 to 1.0 mM O_2/litre-min. Oxygen uptake measurements during the Aspergillus fermentation indicated a peak demand of 0.32 mM O_2/litre-min (Section 6.23) whereas gluconic acid production in the presence of paper pulp (1% wt/wt) indicated an oxygen transfer capability of 0.12 mM O_2/litre-min (Section 7.3) under the most favorable conditions of conventional aeration and agitation that were examined in the bench fermentor.

8.3.12 Power requirements for aeration

The power requirements to supply air at 0.33 V/V/min and 0.033 V/V/min to a fermentor operated at 1 atmosphere head pressure are assumed to be 1 and 0.1 watts/litre, respectively (Chain et al., 1966).

8.3.13 Impeller tip speed

A maximum impeller tip speed of 5.6 m/s is accepted as a suitable design criterion for the agitation system of the large scale fermentor (Holland, 1962). The shear forces resulting from higher impeller tip speeds could damage the mycelia of Aspergillus. Nevertheless, fermentations using Aspergillus are more difficult to stir satisfactorily than fermentations of Penicillium and require higher levels of power input for agitation (Solomons, 1962).

8.3.14 Freeboard allowance

There is to be a freeboard allowance of 25% in the design of the large fermentor to accommodate foam-head, expansion of gassed contents and the cumulative volume of the infusion solutions.

8.3.15 Economic feasibility of the HPC technique

An examination of the economic feasibility of the HPC technique, as it relates to the scale-up of the process described herein, is based on the following assumptions:

(i) A protease titre of 500 mU/ml in the fermentor is required to reach the break-even position relevant to profit and loss when air is used as the sole source of oxygen.

(ii) The fermentation, extraction, purification and packaging stages in the production of protease by Aspergillus oryzae represent basic unit operations that are analogous to those encountered in the production of penicillin by Penicillium chrysogenum.

(iii) The overall process costs, fixed and variable, for the proposed Aspergillus fermentation are similar to those established for the penicillin industry. In 1955 these charges, exclusive of the cost of fermentation medium ingredients, amounted to $1,650. per fermentor of 9000-litre capacity operating on a five-day fermentation cycle.

(iv) The process costs transferred to the Aspergillus fermentation from the Penicillium fermentation are to be adjusted for economy of scale by applying a factor equivalent to the 0.6 power of the capacity ratio of the respective fermentors.

(v) A uniform rate of inflation of 5% per annum is to be applied over the twenty-year period from 1955 to 1975 to obtain contemporary process costs.

8.4 Design Data

8.4.1 Physical dimensions of the bench-scale fermentor:

D_T = 0.21 m

H_L = 0.34 m

D_i = 0.089 m

W = 0.017 m

L = 0.019 m

B = 6

R = 4

J = 0.019 m

I = 2

8.4.2 Operating conditions of the bench-scale fermentor:

$V = 0.012\ m^3$

$\rho = 1020\ kg/m^3$

$\mu = 0.0025\ kg/m\ s$

$K = 1.13\ kg/m\ s^{2-n}$ for 1% paper pulp suspension

$n = 0.42$ for 1% paper pulp suspension

$N = 10\ rev/s$ for conventional aeration-agitation regimen

$Q/V = 0.33\ V/V/min$ for conventional aeration-agitation regimen

$P_0 = 20.3\ HP/1000\ gal$ for conventional aeration-agitation regimen

$P_g = 15.0\ HP/1000\ gal$ for conventional aeration-agitation regimen

$P_g/P_0 = 0.74$

$N = 3.3\ rev/s$ when using HPC infusion technique

$Q/V = 0.033\ V/V/min$ when using HPC infusion technique

$P_0 = 0.82\ HP/1000\ gal$ when using HPC infusion technique

$P_g/P_0 \equiv 1$ when using HPC infusion technique

8.5 Scale-up from the bench fermentor to an industrial fermentor using geometric similarity

Variable	Bench fermentor	Large fermentor
V, m^3	0.012	90.7
D_T, m	0.21	4.13
H_L, m	0.34	6.77
D_i, m	0.089	1.75
W, m	0.017	0.33
L, m	0.019	0.37
B	6	6
R	4	4
I	2	2
J, m	0.019	0.37
D_T/D_i	2.36	2.36
H_L/D_T	1.64	1.64

8.6 Scale-up on the basis of equivalent power per unit volume

The working relationship for scale-up on the basis of constant power per unit volume is:

$$N_1^3 D_{i_1}^2 = N_2^3 D_{i_2}^2 \qquad (1)$$

where subscripts 1 and 2 represent the small and large fermentors, respectively

This identity may be derived from the observation that the Power number is constant under turbulent flow conditions.

$$N_p = P_0 g_c / \rho N^3 D_i^5 = \text{constant}$$

$$\text{thus } P_0 / D_i^5 \propto N^3 D_i^2$$

$$\text{and since } P/V \propto P/D_i^3$$

$$\therefore P/V \propto N^3 D_i^2$$

For conventional aeration-agitation regimen:

$$10^3 \times 0.089^2 = N_2^3 \times 1.75^2 \quad \text{from equation (1)}$$

$$N_2 = 1.37 \text{ rev/s}$$

Similarly for HPC infusion technique at reduced agitation speed:

$$N_2 = 0.46 \text{ rev/s}$$

8.7 Test for impeller power absorbed by the fermentor contents

Richards (1963) arrived at the following formula to predict the horsepower absorbed by turbine-type impellers:

$$HP = 0.095 \times \rho \times N^3 \times D_i^{2.2} \quad W \times L^{1.5} \times B^{0.56} \times R^{0.4} \times J^{0.3}$$

(2).

Substituting design data of the bench fermentor from Section 8.4 above:

$$HP = 0.095 \times 1020 \times 10^3 \times 0.089^{2.2} \times 0.017 \times 0.019^{1.5} \times 6^{0.56} \times$$

$$\times 4^{0.4} \times 0.019^{0.3}$$

$$= 0.095 \times 1020 \times 1000 \times 0.0049 \times 0.017 \times 0.0026 \times 2.73 \times 1.74 \times$$

$$\times 0.30$$

$$= 0.030$$

Power absorbed by both impellers:

$$2 \times 0.030 = 0.060 \text{ HP}$$

Power absorbed per unit volume:

$$(0.060 \text{ HP}/0.012 \text{ m}^3) \times 4.54 \text{ m}^3/1000 \text{ gal} = 22.7 \text{ HP}/1000 \text{ gal}.$$

Similarly at the reduced speed of 3.3 rev/s, the calculated power absorbed per unit volume in the bench fermentor was 0.81 HP/1000 gal.

The measured values for the power absorbed in the bench fermentor, using a torque meter, were 20.3 and 0.82 HP/1000 gal respectively for agitation speeds of 10 and 3.3 rev/s, thus the Richards' correlation can be accepted with reasonable confidence in this scale-up.

The Richards' correlation for power absorbed by each impeller of the large fermentor operating at 1.37 rev/s is as follows from equation (2):

$$HP = 0.095 \times 1020 \times 1.37^3 \times 1.75^{2.2} \times 0.33 \times 0.37^{1.5} \times 6^{0.56} \times$$

$$\times 4^{0.4} \times 0.37^{0.3}$$

$$= 0.095 \times 1020 \times 2.57 \times 3.42 \times 0.33 \times 0.22 \times 2.73 \times 1.74 \times 0.74$$

$$= 217$$

Power absorbed by both impellers:

$$2 \times 217 = 434 \text{ HP}$$

Power absorbed per unit volume:

$$434/20 = 21.7 \text{ HP/1000 gal}$$

Similarly, at the reduced speed of 0.46 rev/s, the power absorbed by each impeller is calculated from equation (2) to be 8.2 HP.

Power absorbed per unit volume:

$$8.2 \times 2/20 = 0.82 \text{ HP/1000 gal}$$

8.8 Calculation of Reynolds numbers as a test for complete mixing of fermentor contents

For fermentation medium without suspended solids:

$$N_{Re} = D_i^2 N \rho/\mu \qquad (3)$$

For fermentation medium with suspended solids exhibiting pseudoplastic flow behaviour:

$$N_{Re'} = \frac{D_i^2 N^{2-n} \rho}{0.1 K \left(\frac{6n+2}{n}\right)^n} \qquad (4)$$

Sample calculations of the Reynolds numbers of the bench fermentor agitated at 10 rev/s with and without suspended solids.

From equation (3) $N_{Re} = \frac{0.089^2 \times 10 \times 1020}{0.0025} = 32{,}232$

From equation (4) $N_{Re'} = \frac{0.089^2 \times 10^{2-0.42} \times 1020}{0.1 \times 1.13 \left[\frac{(6\times0.42)+2}{0.42}\right]^{0.42}} = 1000$

Similarly at the reduced agitation speed of 3.3 rev/s:

N_{Re} = 10,733 for clear fermentation medium

$N_{Re'}$ = 177 for fermentation medium with 1% suspended solids.

Calculations of the Reynolds number for the proposed large fermentor:

$N_{Re} = 1.71 \times 10^{6}$ for clear fermentation medium agitated at 1.37 rev/s

$N_{Re} = 0.57 \times 10^{6}$ for clear fermentation medium agitated at 0.46 rev/s

$N_{Re'}$ = 16,700 for fermentation medium with 1% suspended solids and agitated at 1.37 rev/s

$N_{Re'}$ = 3,000 for fermentation medium with 1% suspended solids and agitated at 0.46 rev/s

Though the modified impeller Reynolds number '$N_{Re'}$' calculated for the bench fermentor barely exceeded the minimum requirements (Section 8.3.5) for complete mixing at the reduced agitation rate, the corresponding calculations for the large fermentor indicated ample turbulence for complete mixing under the least favorable combination of solids-in-suspension and impeller speed.

8.9 Test for impeller tip velocity

Impeller tip velocity is calculated from the expression:

$$\pi N D_i \qquad (5)$$

For the bench fermentor the impeller tip velocity is:

$\pi \times 10 \times 0.089 = 2.8$ m /s when N = 10 rev/s

and

$\pi \times 3.3 \times 0.089 = 0.9$ m /s when N = 3.3 rev/s

For the large fermentor the impeller tip velocity is:

7.5 m /s when N = 1.37 rev/s

and

2.5 m /s when N = 0.46 rev/s

The impeller tip velocity of 7.5 m /s for the large fermentor operating at a power input of 21.7 HP/1000 gal exceeds the design specification of 5.6 m /s given in Section 8.3.13. It is necessary to consider 3 impellers of somewhat smaller diameter on the agitator shaft to permit a reduction in shear rate without sacrificing overall power input to the contents of the fermentor.

8.10 Selection of impeller diameter and rotational speeds for a 3-impeller agitation system

Maxon (1959) indicated that for fermentation vessels the D_T/D_i ratio varies from 3.42 for small, to 2.54 for standard and 2.02 for large impellers.

To minimize shear, an impeller diameter of 1.20 m is selected as the smallest diameter consistent with conventional design practice. This new impeller has a $D_T/D_i = 3.44$.

The corresponding rotational speeds of the 3-impeller shaft for power inputs of 20 HP/1000 gal and 1 HP/1000 gal are calculated from Richards' correlation referred to in Section 8.7 as equation (2).

Rotational speeds of 1.52 and 0.56 rev/s are necessary to provide a power input of 132 and 6.7 HP/impeller.

The three impellers are to be spaced at 1.5 diameters apart as recommended by Richards (1963) to provide good agitation of the entire tank contents and to permit each impeller to make its maximum contribution to power absorption.

8.11 Calculation of Reynolds number and impeller tip velocity for the 3-impeller agitation system

From equation (3)

N_{Re} = 893,000 for clear fermentation medium agitated at 1.52 rev/s

N_{Re} = 329,000 for clear fermentation medium agitated at 0.56 rev/s

From equation (4)

N_{Re} = 9,300 for fermentation medium with 1% suspended solids and agitated at 1.52 rev/s

N_{Re} = 1,900 for fermentation medium with 1% suspended solids and agitated at 0.56 rev/s

From equation (5)

Impeller tip velocity = 5.7 m /s at N = 1.52 rev/s

and

Impeller tip velocity = 2.1 m /s at N = 0.56 rev/s

The values for Reynolds number and impeller tip velocity as re-calculated for a 3-impeller agitation system are acceptable for scale-up implementation.

8.12 Calculation of terminal mixing times

The terminal mixing time factor for single turbine impellers in baffled tanks is given by Norwood and Metzner (1960)

$$N_t = \frac{t\ (ND_i^2)^{2/3}\ g^{1/6}\ D_i^{1/2}}{H_L^{1/2}\ D_T^{3/2}} \qquad (6)$$

For turbulent flow conditions $N_t = 6$

By re-arranging equation (6)

$$t = \frac{6\ H_L^{1/2}\ D_T^{3/2}}{g^{1/6}\ (ND_i^2)^{2/3}\ D_i^{1/2}} \qquad (7)$$

For the case of identical impellers on the same shaft, the liquid depth value 'H_L' is expressed as liquid depth per impeller. Terminal mixing time for the small fermentor agitated at 10 rev/s is:

$$t = \frac{6\ (0.34/2)^{1/2}\ 0.21^{3/2}}{9.81^{1/6} \times 10^{2/3} \times 0.089^{4/3} \times 0.089^{1/2}}$$

$$\frac{6 \times 0.414 \times 0.0955}{1.46 \times 4.65 \times 0.040 \times 0.298} = 2.9\ s$$

Similarly, for the small fermentor agitated at 3.3 rev/s, the terminal mixing time is:

$$t = 6.1\ s$$

For the large fermentor with a 3-impeller agitation shaft, the terminal mixing time when N = 1.52 is:

$$t = \frac{6\ (6.77/3)^{1/2}\ 4.13^{3/2}}{9.81^{1/6}\ \ 1.52^{2/3}\ \ 1.20^{4/3}\ \ 1.20^{1/2}} = 28.3\ s$$

and similarly at the slower speed of 0.56 rev/s the terminal mixing time is:

$$t = 54.9\ s$$

The ratio of mixing times, t_2/t_1 for the reduced agitation level of the large and small fermentors is 9 and within the design specification set out in Section 8.3.7.

8.13 Summary of the design specifications of the large fermentors

Variable	Conventional fermentor	HPC fermentor
V, m^3	90.7	90.7
D_T, m	4.13	4.13
H, m	6.77	6.77
D_i, m	1.20	1.20
W, m	0.33	0.33
L, m	0.37	0.37
B	6	6
R	4	4
I	3	3
J, m	0.37	0.37
N, rev/s	1.52	0.56
HP/1000 gal (ungassed)	20	1
N_{Re} (clear medium)	893×10^3	329×10^3
N_{Re} (mold solids)	9.3×10^3	1.9×10^3
t, s	28.3	54.9
Q, m^3/min (max)	30.2	3.0

8.14 Energy requirements for the agitation-aeration of the conventional and HPC fermentors

8.14.1 Conventional fermentor: five-day fermentation cycle

Time, h	Aeration rate, V/V/min	P_g/P_0	Agitation power, HP	Power absorbed, watts/litre		
				Aeration	Agitation	Total
72	0.033	1.0	396	0.1	3.26	3.36
48	0.33	0.74	293	1.0	2.41	3.41

Total energy consumption for aeration-agitation per fermentation:

$$3.36 \times 90,700 \times 72 \times 10^{-3} + 3.41 \times 90,700 \times 48 \times 10^{-3} = 36,787 \text{ kWh}$$

8.14.2 HPC fermentor: four-day fermentation cycle

Time, h	Aeration rate, V/V/min	P_g/P_0	Agitation power, HP	Power absorbed, watts/litre		
				Aeration	Agitation	Total
96	0.033	1.0	20	0.1	0.16	0.26

Total energy consumption for aeration-agitation per fermentation:

$$0.26 \times 90,700 \times 96 \times 10^{-3} = 2,264 \text{ kWh}$$

8.15 Estimation of the infusion rates of the HPC solutions

It is necessary to supply oxygen at the rate of 0.24 mM O_2/litre-min. The infusion solutions are assumed to be solutions of 50% H_2O_2 and a solution of beef liver catalase, standardized at 100,000 Keil units/litre.

8.15.1 Infusion rate of H_2O_2 to supply 0.24 mM O_2/litre-min

Oxygen requirement = $90,700 \times 0.24 \times 10^{-3} \times 32 = 697$ g O_2/min

H_2O_2 requirement = $697 \times 34/16 = 1481$ g H_2O_2/min

1 ml 50% H_2O_2 contains 0.598 g 100% H_2O_2

50% H_2O_2 requirement = $1481 \times 1/0.598 \times 1/1000 = 2.48$ litres/min

The H_2O_2 infusion pump should be capable of metering over the range of 1-10 litres/min.

8.15.2 Infusion rate of catalase solution to supply 0.24 mM O_2/litre-min

To provide an excess of catalase to prevent the accumulation of hydrogen peroxide, the catalase infusion rate is to be 1.5 times that indicated from the Keil activity.

Catalase requirement = $1481 \times 1.5 = 2222$ Keil units/min

Catalase activity = 100,000 Keil units/litre

Catalase infusion rate = $2222/100,000 = 0.022$ litres/min

The catalase infusion pump should be capable of metering over the range of 10-100 ml /min

8.16 Cost estimations of H_2O_2 and catalase material consumed during a fermentation

8.16.1 Cost of hydrogen peroxide, 50%

Volume required for 24 h = $2.48 \times 60 \times 24$ = 3571 litres

3571 litres weights 7857 lbs

Cost of H_2O_2 at \$25/cwt = $.7857 \times 25/100$ = \$1,964/fermentation

8.16.2 Cost of catalase, 100,000 Keil units/litre

Volume required for 24 h = $0.022 \times 60 \times 24$ = 31.7 litres

Cost of catalase at \$63/litre = 31.7×63 = \$1,996/fermentation

8.16.3 Cost comparison of HPC and conventional agitation-aeration techniques

The cost of hydrogen peroxide and catalase materials to supply oxygen at the rate of 0.24 mM O_2/litre-min for a sustained time interval of 24 hours is \$3,960. The reduction in energy consumption by 34,500 kWh when compared to the operation of a conventional fermentation, represents a saving of approximately \$690; if charges for electrical energy are assumed to be 2¢/kWh at a power factor > 90%.

The estimated cost of \$3,960 for the HPC materials is substantially greater than the expected saving of \$690 in electrical energy. The net deficit of \$3,270 per fermentation would have to be offset by additional fermentation productivity to warrant utilization of the HPC system for adding oxygen.

8.17 Examination of the economic feasibility of the HPC oxygenation technique

The economic feasibility of the HPC technique is examined in terms of the additional protease titre necessary to compensate for the net deficit of $3,270 per fermentation. It is assumed that a protease titre of 500 mU/ml is required to offset all process costs when oxygen is added to the fermentation by conventional aeration techniques.

8.17.1 Cost of medium ingredients per 90,700-litre fermentation

The ingredients of the standard nitrate-free fermentation medium are listed in Table 43, Section 6.19.

2,050 kg sugar @$0.75/kg	$1,537.
2,050 kg phosphate salts @$0.50/kg	1,025.
365 kg lactic acid @$1.00/kg	365.
182 kg succinate @$4.00/kg	728.
water, antifoam and trace minerals	45.
Total	$3,700.

8.17.2 Estimation of overall process costs, exclusive of medium ingredients and HPC materials

In 1955 the break-even process charge for penicillin production, exclusive of fermentation medium ingredients, amounted to $1,650. per 9,000-litre fermentation. The comparable process charge, corrected for scale and inflation,

that would apply to the proposed Aspergillus fermentation is as follows:

$$\$1,650 \times \left(\frac{90,700}{9,000}\right)^{0.6} \times e^{(1975-1955)0.05} =$$

$$\$1,650 \times 4.0 \times 2.7 = \$17,820.$$

8.17.3 Total process costs for the Aspergillus fermentation utilizing the conventional aeration-agitation technique

Cost of medium ingredients	$3,700.
Remaining process costs	17,820.
Total process costs	$21,520.

8.17.4 Total process costs for Aspergillus fermentation utilizing the HPC technique as a source of oxygen

Net cost of HPC materials	$3,270.
Cost of medium ingredients	3,700.
Remaining process costs	17,820.
Total process costs	$24,790.

8.17.5 Estimation of the additional protease required to warrant the use of the HPC system for adding oxygen

The net cost of HPC materials increased the total process costs of a conventionally aerated-agitated Aspergillus fermentation by

$$\frac{\$3,270}{\$21,500} \times 100 = 15.2\%$$

On the assumption that a protease titre of 500 mU/ml is required to cover processing costs of a conventional fermentation, an additional protease titre of 76 mU/ml (i.e. 500 × 15.2%) is required to warrant the use of the HPC system for adding oxygen.

8.17.6 Discussion

As indicated earlier, it is unlikely that the HPC oxygenation technique will replace conventional agitation-aeration techniques in the fermentation industry except in specific instances when a significant improvement in yield can be demonstrated which will more than offset the additional cost of raw materials. This improvement in yield might reflect the ability of the HPC technique to satisfy high oxygen demands of the culture during critical phases in the fermentation cycle or the ability to permit precise control of the oxygen tension at any pre-determined value.

In addition to the possibility of improved yields, the use of the HPC technique as a source of oxygen would permit increased utilization of existing fermentation capacity due to a marked reduction in surface foam and a reduction in the air-induced expansion of the liquid contents of the fermentor. The standard freeboard allowance of 25% of the volume of the fermentor could be reduced to 10%, in all probability.

Finally, there is the prospect that the improved supply of oxygen through the use of the HPC technique will appreciably shorten the fermentation cycle, thereby increasing the frequency of consecutive fermentations. A decrease in an overall fermentation cycle of six days (five days fermentation and one day turn-around) to five days represents an increase of 17% in productivity.

APPENDIX 1

ANALYSIS OF VARIANCE

The data in the analysis matrix below show protease titres of Aspergillus oryzae in response to changes in fermentation parameters; the details of which are described in Section 4.10. This was a three-factor experiment with interaction in which there were two classes of row factors (R_1 & R_2), three classes of column factors (C_1, C_2 & C_3) and two classes of group factors (G_1 & G_2), wherein:

R_1 = 1 ml spore inoculum

R_2 = 5 ml spore inoculum

C_1 = 0 ppm Alpha-floc

C_2 = 390 ppm Alpha-floc

C_3 = 1800 ppm Alpha-floc

G_1 = autoclave 3 min

G_2 = autoclave 30 min

Analysis Matrix:

	C_1		C_2		C_3		
	G_1	G_2	G_1	G_2	G_1	G_2	
R_1	287 475 762	480 492 972	492 427* 919	472 451 923	375* 427 802	451 914 865	T_{R_1} 5243
R_2	398 373 771	459 451 910	443 328 771	410 398 808	336 369 705	328 303 631	T_{R_2} 4596
	1533	1882	1690	1731	1507	1496	9839
	T_{C_1} 3415		T_{C_2} 3421		T_{C_3} 3003		

$T_{G_1} = 4730$ $T_{G_2} = 5105$

*average of the remaining 7 titres from the appropriate column.

Additional Notation

T = total
c = number of column classes = 3
r = number of row classes = 2
g = number of group classes = 2
n = number of replications = 2
N = number of assay results
$\sum X^2$ = summation of squares of all assay results.

Sum of Squares

(i) Among columns, $SS_C = \frac{\sum T_G^2}{nrg} - \frac{T^2}{N}$

$$= \frac{(3415)^2 + (3421)^2 + (3003)^2}{2 \times 2 \times 2} - \frac{9839^2}{24} = \underline{14354}$$

(ii) Among rows, $SS = \frac{\sum T_R^2}{ncg} - \frac{T^2}{N}$

$$= \frac{(5243)^2 + (4596)^2}{2 \times 3 \times 2} - \frac{9839^2}{24} = \underline{17442}$$

(iii) Among groups, $SS = \frac{\sum T_G^2}{ncr} - \frac{T^2}{N}$

$$= \frac{(4730)^2 + (5105)^2}{2 \times 3 \times 2} - \frac{9839^2}{24} = \underline{5985}$$

(iv) Column-Row interaction, $SS = \frac{\sum T_{CR}^2}{ng} - \frac{T^2}{N} - SS_C - SS_R$

$$= \left[(762+972)^2 + (919+923)^2 + (802+965)^2 + (771+910)^2 + (771+808)^2 + (705+631)^2\right] \div 2 \times 2 - \frac{9839^2}{24}$$

$$- 14354 - 17442 = \underline{5251}$$

(v) Row-Group interaction, $SS = \frac{\sum T_{RG}}{nc} - \frac{T^2}{N} - SS_R - SS_G$

$= \left[(762+919+802)^2 + (972+923+865)^2 \right.$

$\left. + (771+771+705)^2 + (910+808+631)^2\right] \div 2 \times 3$

$- \frac{9839^2}{24} - 17442 - 5985 = \underline{1276}$

(vi) Column-Group interaction, $SS = \frac{\sum T^2_{CG}}{nr} - \frac{T^2}{N} - SS_C - SS_G$

$= \left[(762+771)^2 + (972+910)^2 + (919+771)^2 + (923+808)^2 \right.$

$\left. + (802+705)^2 + (865+631)^2\right] \div 2 \times 2 - \frac{9839^2}{24}$

$- 14,354 - 5985 = \underline{9466}$.

(vii) Column-Row-Group interaction, $SS = \frac{\sum T^2_{CRG}}{n} - \frac{T^2}{N} - SS_C$

$- SS_R - SS_G - SS_{CR} - SS_{RG} - SS_{CG}$

$= \left[762^2+972^2+919^2+923^2+802^2+865^2+771^2+910^2+771^2 \right.$

$\left. + 808^2 + 705^2 + 631^2\right] \div 2 - \frac{9839^2}{24} - 14354 - 17442$

$- 5985 - 5251 - 1276 - 9466 = \underline{1834}$

(viii) $\text{Total SS} = \sum X^2 - \frac{T^2}{N}$

$$= 287^2+475^2+480^2+492^2+398^2+373^2$$

$$+ 459^2+451^2+492^2+427^2+443^2+328^2$$

$$+ 472^2+451^2+410^2+398^2+375^2+427^2$$

$$+ 336^2+369^2+451^2+414^2+328^2+303^2$$

$$- \frac{9839^2}{24} = \underline{85,609}$$

Analysis of Variance: Summary with Computations

	Sum of Squares	Degrees of Freedom	Mean* Square	Variance** Ratio	F 0.95	F 0.99
Columns	14,354	c-1 = 2	7,177	2.9	3.89	6.93
Rows	17,442	r-1 = 1	17,442	7.0	4.75	9.33
Groups	5,985	g-1 = 1	5,985	2.4	4.75	9.33
C-R	5,251	(c-1)(r-1) = 2	2,626	1.1	3.89	6.93
R-G	1,276	(r-1)(g-1) = 1	1,276	0.5	3.89	6.93
C-G	9,466	(c-1)(g-1) = 2	4,733	1.9	4.75	9.33
C-R-G	1,834	(c-1)(r-1)(g-1) = 2	917	0.4	3.85	6.93
Residual	30,001	12	2,500			
Total	85,609	N-1 = 23				

* Mean square $= \dfrac{\text{sum of squares}}{\text{degrees of freedom}}$

** Variance ratio $= \dfrac{\text{mean square of variable}}{\text{mean square of residual}}$

APPENDIX 2

CORRELATION COEFFICIENT

X pH	Y protease, mU/ml	Y_1 Y-200	X^2	Y_1^2	XY_1
6.85	533	333	46.92	110,889	2,281.05
6.80	467	267	46.24	71,289	1,815.60
6.75	471	271	45.56	73,441	1,829.25
6.75	455	255	45.56	65,025	1,721.25
6.50	398	198	42.25	39,204	1,287.00
6.50	385	185	42.25	34,225	1,202.50
6.45	394	194	41.60	37,636	1,251.30
6.50	402	202	42.25	40,804	1,313.00
6.30	197	-3	39.69	9	-18.90
6.35	266	66	40.32	4,356	419.10
6.25	144	-56	39.06	3,136	-350.00
6.25	168	-32	39.06	1,024	-200.00
6.15	209	9	37.82	81	55.35
6.10	193	-7	37.21	49	-42.70
6.00	140	-60	36.00	3,600	-360.00
6.15	172	-28	37.82	784	-172.20

$\sum X = 102.65$ $\sum Y_1 = 1794$ $\sum X^2 = 659.61$ $\sum Y_1^2 = 485,552$ $\sum XY_1 = 12,031.60$

$\frac{\sum X}{n} = 6.42$ $\frac{\sum Y_1}{n} = 112.12$ $\frac{\sum X^2}{n} = 41.23$ $\frac{\sum Y_1^2}{n} = 30,347$ $\frac{\sum XY_1}{n} = 751.98$

Covariance: $$s^2_{xy_1} = \sum \frac{XY_1}{n} - \left(\frac{\sum X}{n} \times \frac{\sum Y_1}{n}\right)$$

$$= 751.98 - (6.42 \times 112.12) = 32.58$$

Standard deviation: $$s_x = \sqrt{\frac{\sum X^2}{n} - \left(\frac{\sum X}{n}\right)^2}$$

$$\sqrt{41.23 - 6.42^2} = 0.247$$

Standard deviation: $$s_{y_1} = \sqrt{\frac{\sum Y_1^2}{n} - \left(\frac{\sum Y_1}{n}\right)^2}$$

$$= \sqrt{30,347 - 112.12^2} = 133.3$$

Correlation coefficient: $$\frac{s^2_{xy_1}}{s_x s_{y_1}}$$

$$= \frac{32.58}{133.3 \times 0.247} = 0.99$$

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